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Poster Presentation

FASEB/ASBMB Symposium - April, 1991
Poster # 2947

The Rapid Isolation of Highly Purified Cadmium Metallothioneins Using HPLC

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INTRODUCTION:

The central role of the proximal tubule in the renal toxicity due to cadmium (Cd) poisoning has been well documented. However, the mechanisms leading to the onset of this nephrotoxic effect are presently not well understood. The most likely mechanism for the initiation of toxicity involves the accumulation of Cd in the liver where it induces the formation of, and is complexed with the metallothioneins (MT-I and MT-II), low molecular weight binding proteins, followed by release from the liver and subsequent absorption by the proximal tubule. Methods for obtaining MTs for specific RIA and immunohistochemistry have been hampered by difficulties in removing impurities, particularly from the more immunogenic of the two Cd-binding proteins, Cd-MT-I. To obtain highly purified Cd-MTs for use in RIA as well as to assay the relative affects of exposure to Cd-MT and ionic Cd in vitro on human proximal tubule cells, Cd-MT-I and Cd-MT-II were prepared from rat liver for use in these studies using a two step chromatographic procedure employing traditional gel filtration followed by a rapid high performance anion exchange chromatographic step. The two main fractions collected were assessed for homogeneity by analytical anion exchange, gel filtration (GFC) and reverse phase high performance chromatography. The fractions were also assayed for cadmium by atomic absorbance spectroscopy and by high performance liquid chromatography.

SAMPLE PREPARATION:

Liver homogenates containing Cd-MT-I and Cd-MT-II were prepared as described by Winge *et al* (1). Essentially, rats injected subcutaneously with cadmium chloride were sacrificed, the livers excised and pooled and homogenized with 0.001 M potassium phosphate buffer, pH 7.8. The homogenates were centrifuged to yield particle-free supernatant fluids which were heated at 60° C for 10 minutes. The resulting coagulated material was removed by centrifugation. The supernatant fluids were concentrated by lyophilization and chromatographed on a Sephadex G-75 column eluted with 0.01 M potassium phosphate buffer, pH 7.8. The cadmium containing fractions were pooled and further purified by high performance anion exchange chromatography.

CHROMATOGRAPHIC RESULTS:

Figure 1 shows a high performance anion exchange chromatogram in which the real-time absorbance ratio's (250nm/280nm) of 24-28 (higher than previously reported) were used to differentiate Cd-MT-I and Cd-MT-II from other proteins present in the sample. Repeated anion exchange separations revealed a time dependent conversion of a non-metallothionein protein component to form which coeluted with Cd-MT-1 (Figure 2). The formation of the interfering protein was eliminated by keeping the sample at refrigerated temperatures and utilizing a fast high performance anion exchange purification step. Figure 3 illustrates the preparative purification of the crude material in which two fractions corresponding to Cd-MT-I

and Cd-MT-II were collected. The overall time for this purification step was 30 minutes. Figures 4-6 show the chromatographic evaluation of the collected fractions by anion exchange, gel filtration and reverse phase high performance liquid chromatography. In order to determine the concentration of cadmium present in the collected fractions, samples of each were submitted for analysis by atomic absorption spectroscopy as well as HPLC analysis for transition metals. Figure 7 shows the separation of nine standard transition metals by HPLC. The fractions as well as the crude GFC material were first diluted with 50 mM nitric acid followed by passage through a C_{18} Sep-Pak® Plus cartridge. Analysis of the fractions demonstrated the presence of cadmium as well as zinc (Figure 8). These results (Figure 8) correlate well with those observed with AA and show a constant cadmium/zinc ratio with the anion exchange purification step.

CONCLUSIONS:

The anion exchange purification methodology has demonstrated the importance of speed which has permitted the isolation of highly purified Cd-MT's in high yields. Due to the sensitivity, this method may also be amenable as an alternative for a non-radioactive assay of Cd-Mt formation and breakdown in toxicological studies. It is also demonstrated that the HPLC analysis of transition metals is consistent with that of AA and additional information can be obtained in a single run.

REFERENCES:

1. Winge, D.R., Premakumar, R., and Rajagopalan, V.K. (1975) *Arch. Biochem. Biophys.* **170**, 242-252.

CHROMATOGRAPHIC CONDITIONS

ANALYTICAL ANION EXCHANGE CHROMATOGRAPHY

Column:	Protein-Pak™ DEAE 8HR in a glass AP-1 column (10 x 100 mm)
Method:	Auto•Blend™ Tris
Initial Conditions:	20 mM Tris, pH 8.5
Final Conditions:	20 mM Tris, pH 8.5 plus 50 mM Sodium Chloride
Gradient Duration:	20 minutes, linear
Flow Rate:	1.5 ml/min
Detection:	250 nm, 280 nm and Ratio 250/280
Instrumentation:	650 Advanced Protein Purification System and 490 Programmable Multi-Wavelength UV/Visible Detector

PREPARATIVE ANION EXCHANGE CHROMATOGRAPHY

Column:	Protein-Pak DEAE 8 HR in a glass AP-5 column (50 x 100 mm)
Method:	Auto•Blend Tris (as above)
Flow Rate:	37.5 ml/min.
Detection:	250 nm
Injection Vol.:	50 ml
Instrumentation:	650 Advanced Protein Purification System and 484 Tunable UV/Visible Detector with a non-metallic flow cell

REVERSE PHASE CHROMATOGRAPHY

Column:	Delta-Pak™ HPI (3.9 x 150 mm) C_{18}, 5 µm, 300Å
Eluent A:	Water with 0.1% TFA
Eluent B:	Acetonitrile with 0.1% TFA
Gradient:	0% B to 40% B in 30 minutes (linear) hold for 5 minutes
Flow Rate:	0.5 ml/min.
Column Temp.:	30° C
Detection:	214 nm
System:	625 Non-Metallic HPLC System with 994 Photodiode Array Detector

GEL FILTRATION CHROMATOGRAPHY

Columns:	Protein KW-802.5, 803, and 804
Eluent:	50 mM Sodium Phosphate, pH 7.0 with 300 mM NaCl
Flow Rate:	0.5 ml/min
Detection:	250 nm
System:	650 Advanced Protein Purification System and 490 Programmable Multi-Wavelength UV/Visible Detector

TRANSITION METAL ANALYSIS CHROMATOGRAPHY

Column:	Delta-Pak C_{18}, 5 µm, 100Å (3.9 x 150 mm)
Eluent:	2 mM Sodium Octane Sulfonate and 35 mM Tartrate, pH 3.65 with 5% Acetonitrile
Flow Rate:	0.8 ml/min
Detection:	Post-column PAR at 0.4 ml/min, monitored at 500 nm
System:	ActION Analyzer Non-Metallic System, Reagent Delivery Module and 490 Programmable Multi-Wavelength UV/Visible Detector

Methods Development - Anion Exchange Chromatography of Crude Material

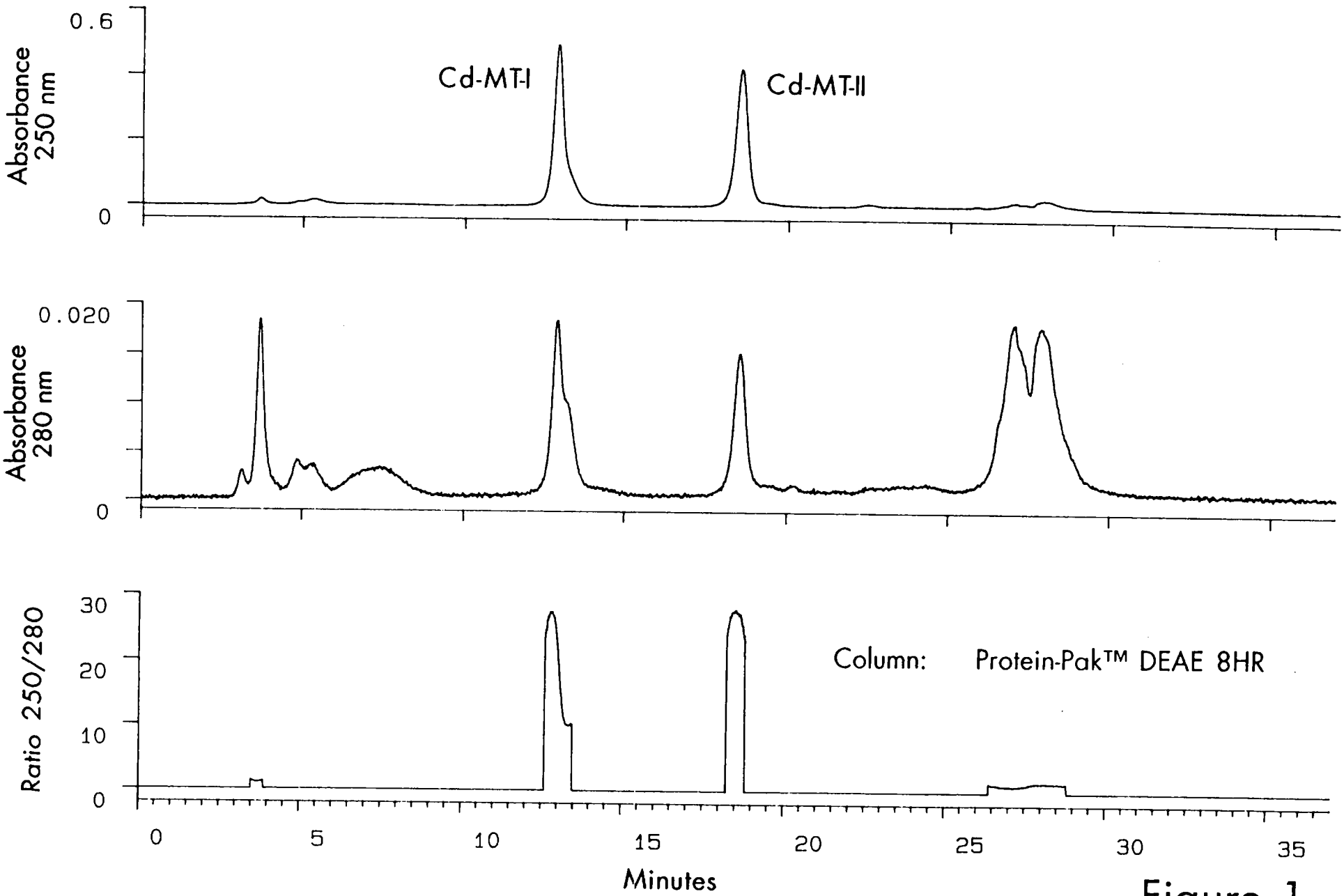


Figure 1

Analysis of Sample Degradation by Anion Exchange Chromatography

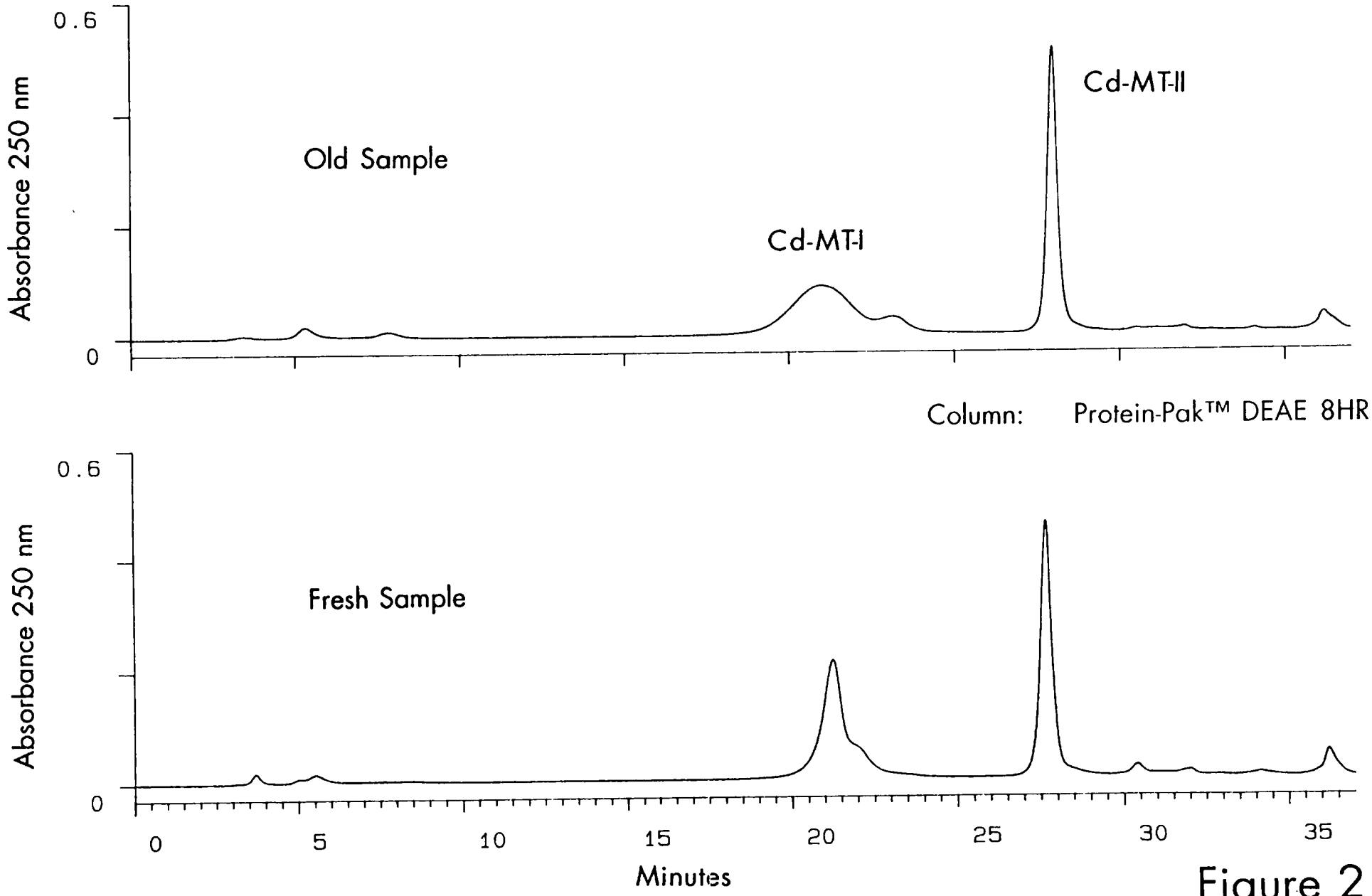


Figure 2

Preparative Anion Exchange Chromatography

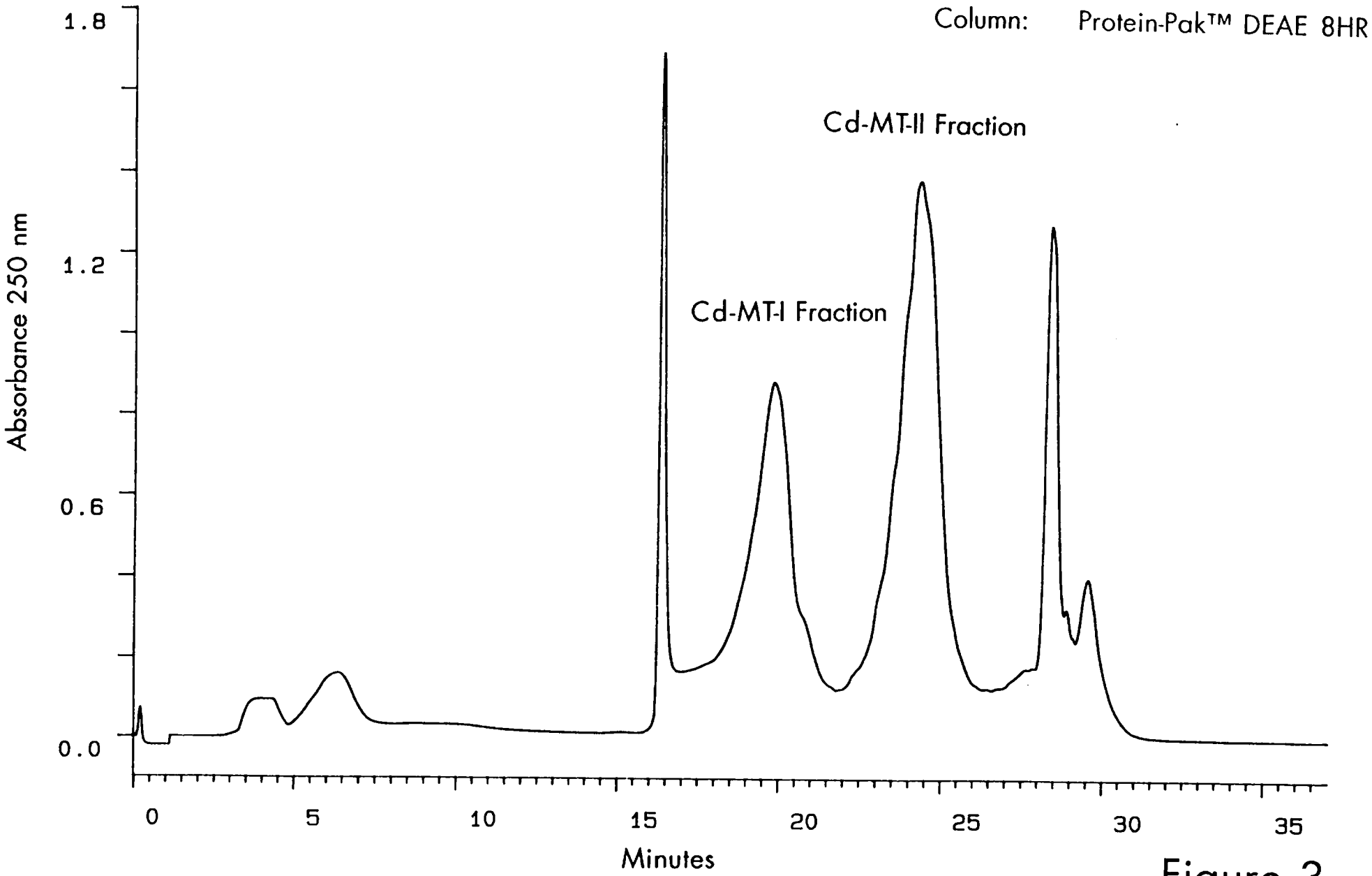


Figure 3

Analysis of Fractions by Anion Exchange Chromatography

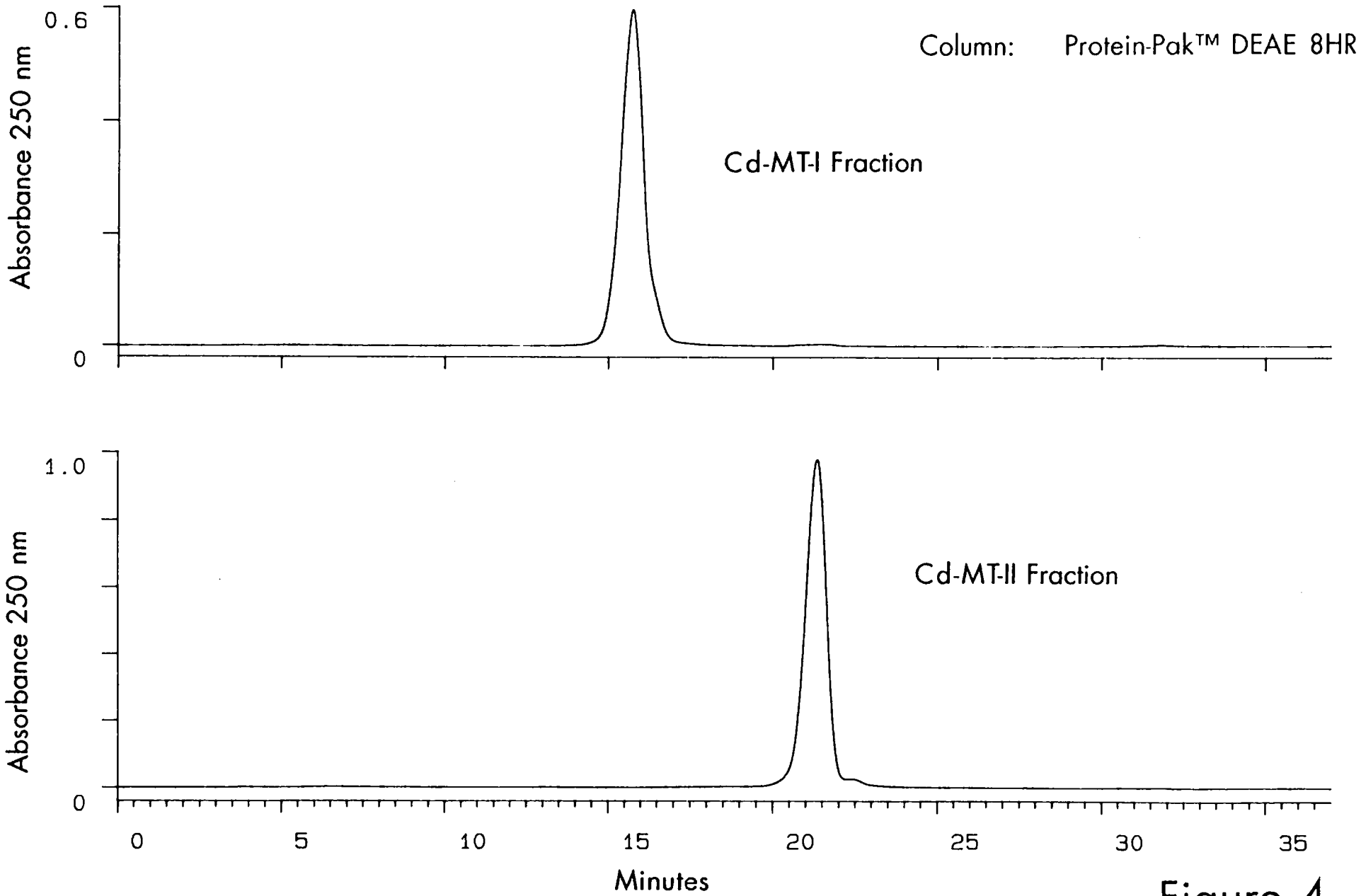


Figure 4

Analysis of Fractions by Gel Filtration Chromatography

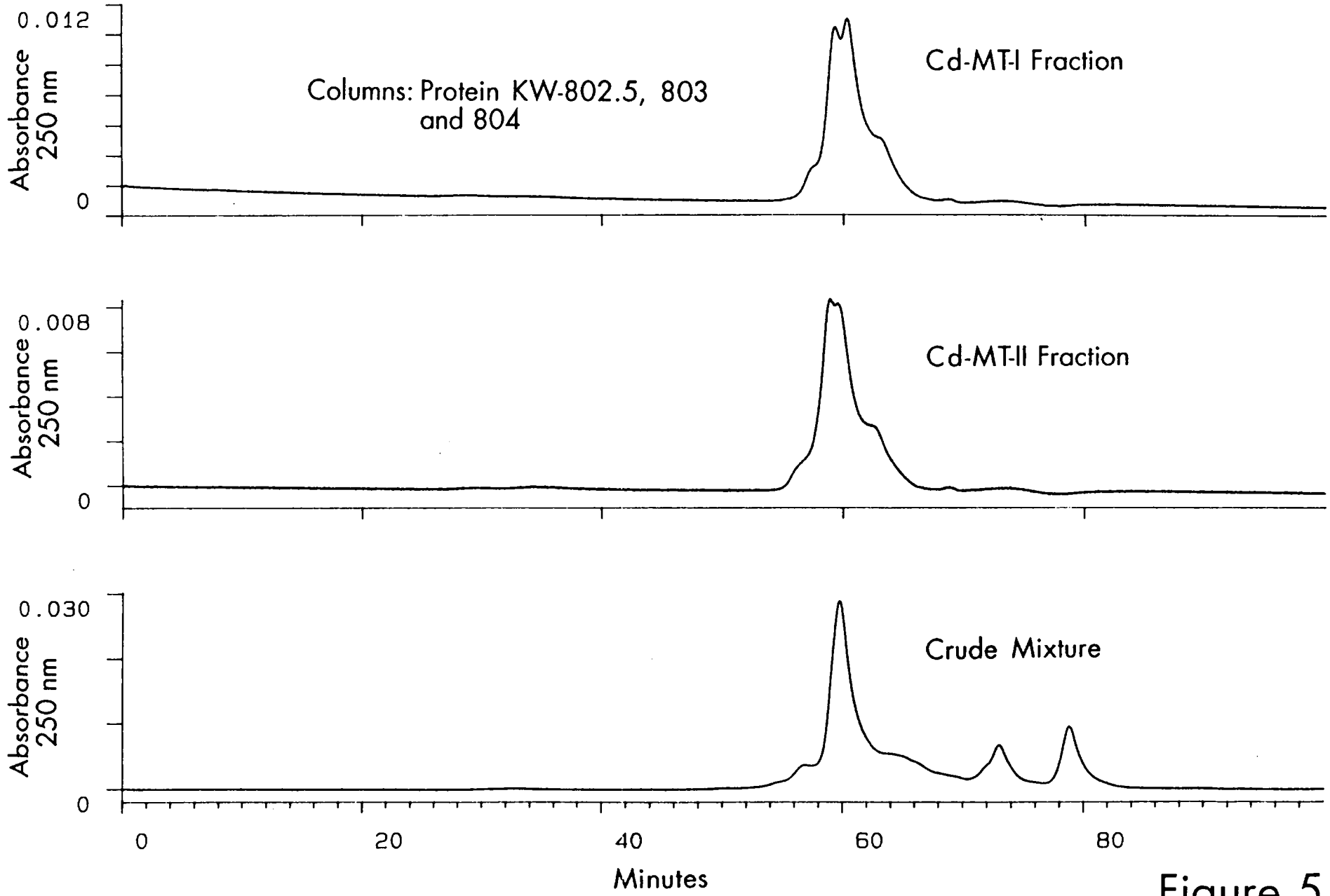


Figure 5

Analysis of Fractions by Reverse Phase Chromatography

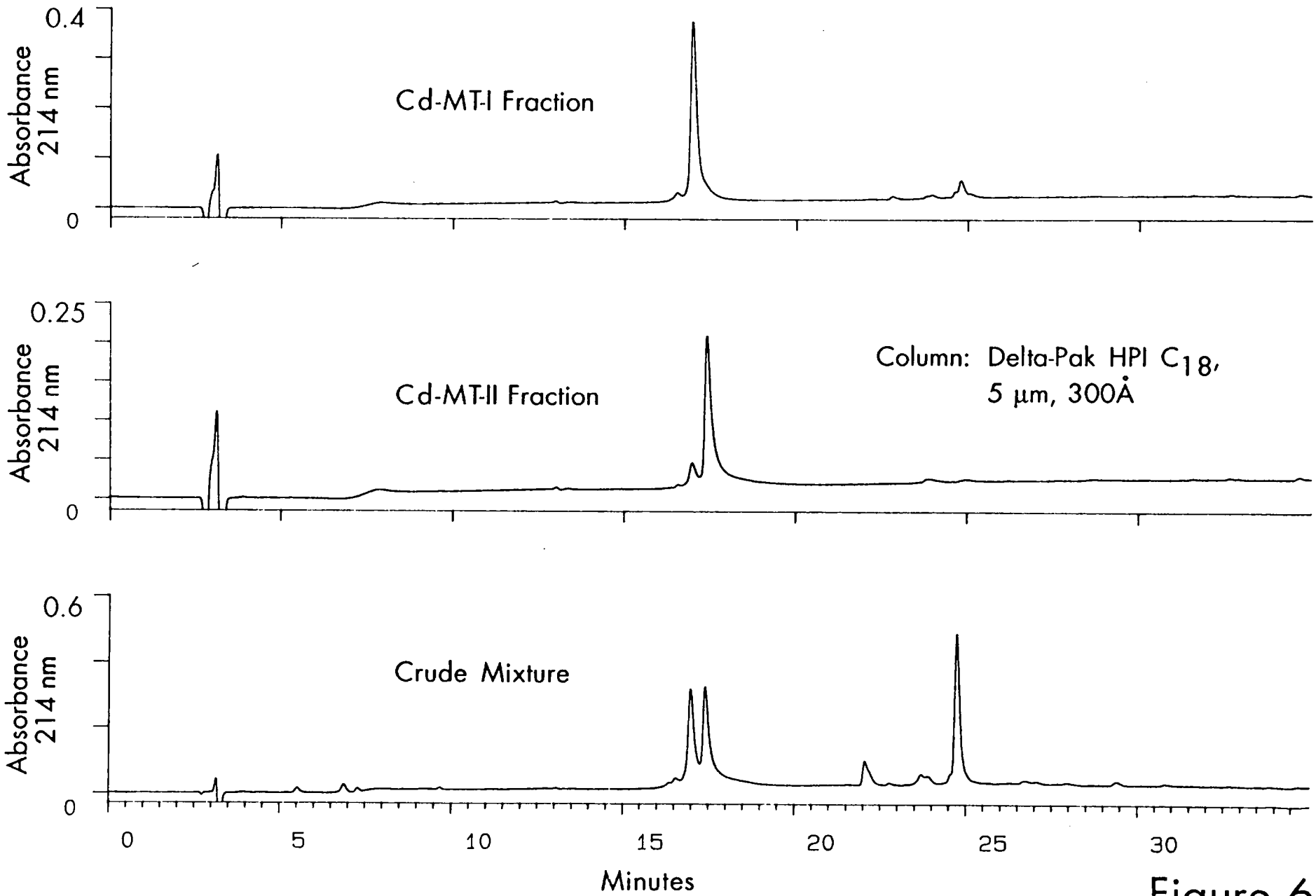


Figure 6

Transition Metal Analysis by Reverse Phase Chromatography

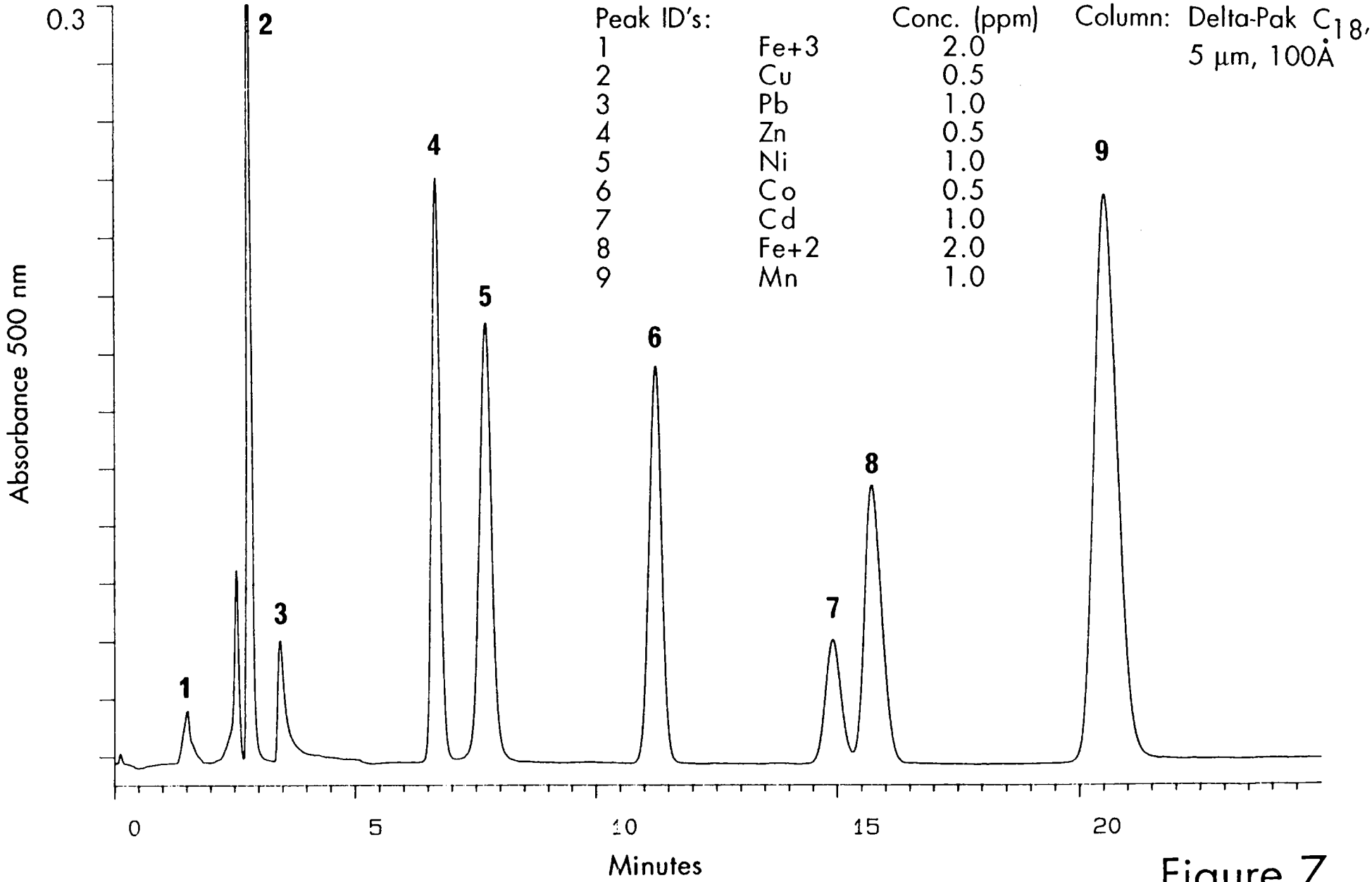
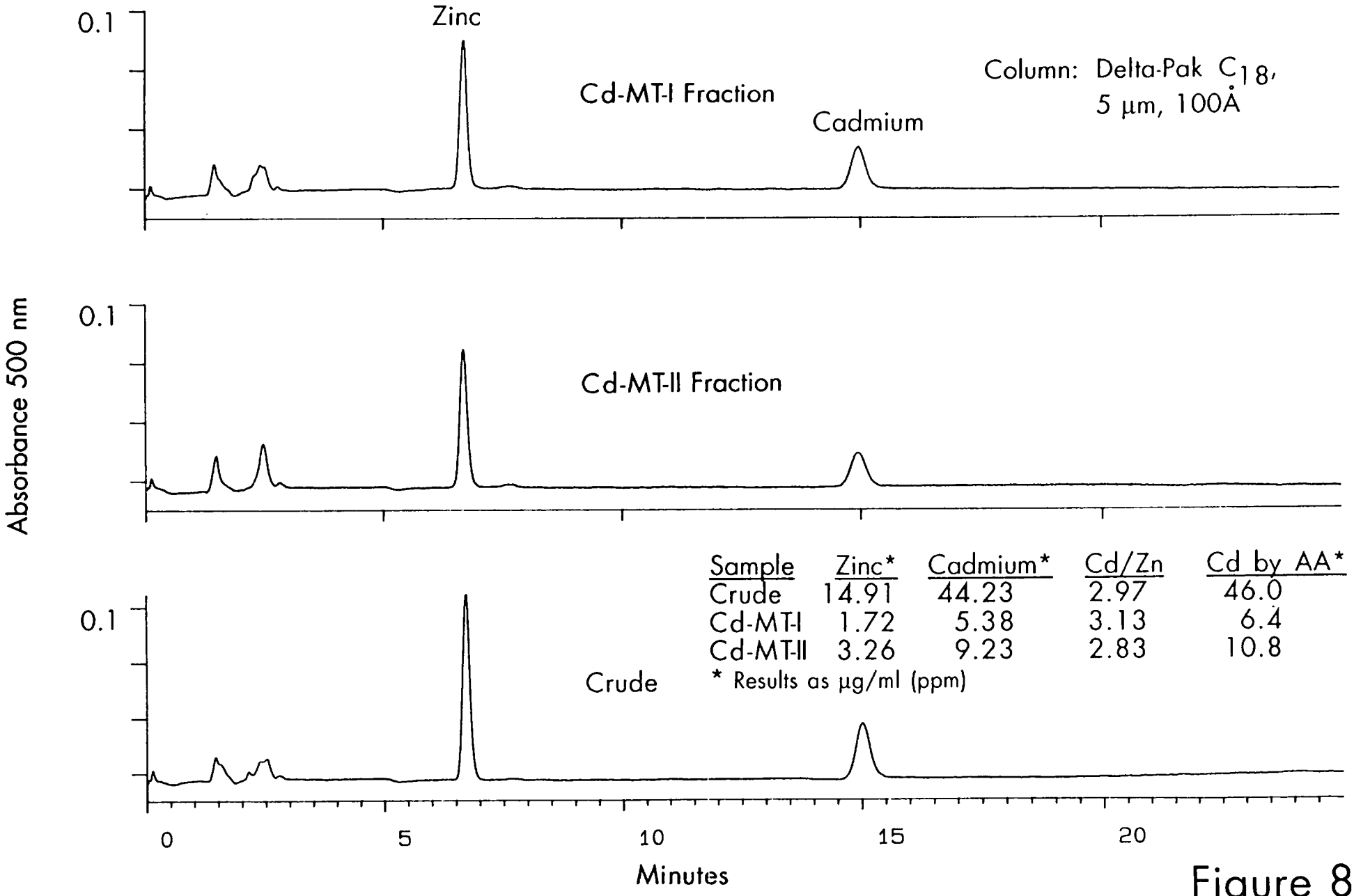


Figure 7

Transition Metal Analysis of Fractions and Crude



Sample	Zinc*	Cadmium*	Cd/Zn	Cd by AA*
Crude	14.91	44.23	2.97	46.0
Cd-MT-I	1.72	5.38	3.13	6.4
Cd-MT-II	3.26	9.23	2.83	10.8

* Results as µg/ml (ppm)

Figure 8

ANALYSIS OF TRANSITION METALS USING DYNAMICALLY COATED REVERSE PHASE COLUMNS AND POST COLUMN DERIVITAZATION

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508/478-2000 x3043

ANALYSIS OF TRANSITION METALS USING DYNAMICALLY COATED REVERSE PHASE COLUMNS AND POST COLUMN DERIVATIZATION

Work by Cassidy in the late 1970's showed that HPLC reverse phase columns could be modified with alkyl sulfonate salts to yield columns with cation exchange character. Reverse phase columns generally exhibit higher column efficiencies than cation exchange resins. This increased efficiency gives sharper, narrower peaks and hence better detection limits. The dynamically coated concept is used to separate divalent transition metals such as Mn, Fe, Co, Ni, Cu, Zn, Cd, and Pb.

Octane sulfonate (OS) present in the eluent will saturate into the column after a short equilibration time. Since it is not permenantly bonded to the column, any loss of OS with time and due to the sample matrix will be replenished by the eluent. The concentration of the OS can be varied from 1 to 10mM to meet the cation capacity needs of the sample matrix. Data shows that retention of transition metals increase with increasing OS concentrations.

Normally cations are eluted from cation exchange columns using H+ or other cations competing for the site. This works well for alkali and alkaline earth cations but is not effective for transition metals. However, transition metals will chelate with various organic acids making them anionic or neutral metal complexes. Transition metal retention and elution is dependant upon the equilibrium between free and complexed metal. This equilibrium can be shifted by changing the pH and organic acid concentration. Generally, metal retention is inversely related to the metal-organic acid binding strength, concentration of organic acid, and eluent pH. Tartrate is the organic acid of choice but others can be used with an expected change in selectivity.

The use of reverse phase columns with OS gives a secondary ion interaction separation mechanism commonly referred to as ion pairing reverse phase. In this mechanism the use of organic solvent inversely effects retention. For dynamically coated transition metal separations, a small addition of acetonitrile improves column efficiency and resolution with a net decrease in retention.

Using these 4 retention/separation variables, the above transition metals can be resolved on a Waters Delta-Pak C18 column (4.9mm x 15cm, 100°A, 5µ particle, endcapped) using 2mM NaOS / 35mM Tartrate / pH=3.65 / 5% AcCN within 20minutes.

After the transition metals are separated they are derivatized post column with PAR. PAR displaces the organic acid and forms metal-PAR complex which has significant absorption properties between 490 and 550nm. PAR is specific for transition metals; alkali and alkaline earth cations, although retained on the column, do not react with PAR nor interfere with PAR-metal formation. The response for transition metals is linear between 10ppb to 5ppm using a 100µL injection.

Several Waters UV/Visible detectors were evaluated for response at 500nm. The Waters 490 detector with Xe lamp was shown to give 25% more response than the Waters 484 with deturium lamp between 500 and 546 nm and 68% more respone than the Waters 440 with Hg lamp at 546nm. Using the 490 detector at 520nm the detection limit using a 100L injection for Cu, Zn, Ni, Co, and Mn is 10ppb and 25ppb for Fe+3, Fe+2, Pd, and Cd.

The Waters Transition Metal method has been successfully applied to high acid matrices (sample pH 1), high salt (-up 100mM NaClO4), high organic matices such as fermentation brooths and dry ashed milk, and samples that have disparate levels of transtion metals such as plating baths.

With this method iron+3 elutes in the void where quantitation may be comprimized. Adding ascorbic acid to the sample reduces the iron+3 to iron+2, a well retained peak free of interferences, to give total iron concentration. Iron +3 can be quantitated as the difference between total iron and iron+2.

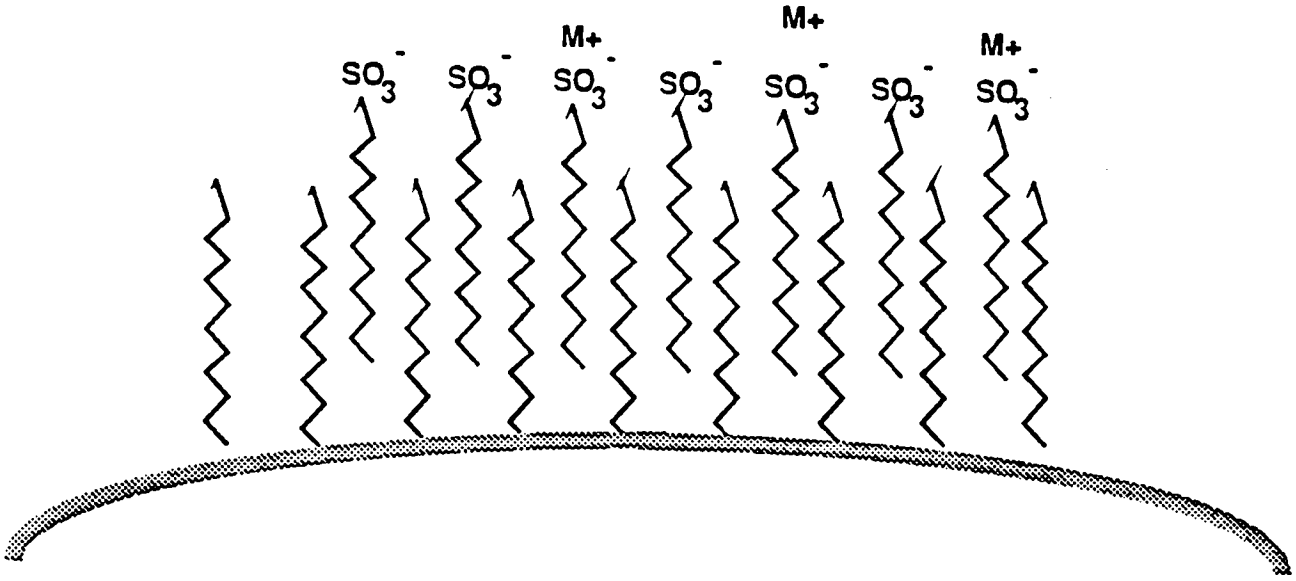
Neutral organics present in 18 megohm water will effect iron+3 and copper peak shape and quantitation. These organics are concentrated from the eluent at the head of the column over time and show increasing distortion of copper peak shape. The column can be washed with 50% acetonitrile / water to eliminate the problem; but it will reoccur. Placing a C18 scrubber column between the pump and the injector will remove the organics and give better long term peak shape and reproducibility.

DYNAMICALLY COATED REVERSE PHASE COLUMN TRANSITION METAL RETENTION

Reverse phase C18 columns are saturated with octane sulfonate to produce a column with cation exchange character.

The cation capacity can be increased with increased octane sulfonate in the eluent.

In octane sulfonate only eluent, transition metal are strongly retained.



DYNAMICALLY COATED REVERSE PHASE COLUMN TRANSITION METAL ELUTION

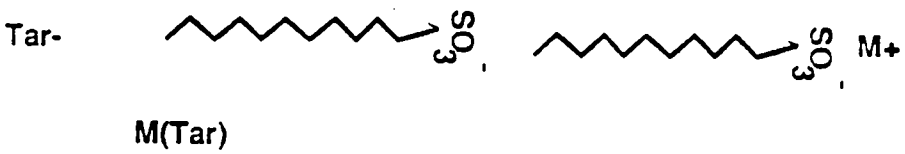
Transition metals form neutral and/or anionic complexes with organic acids such as tartrate. These complexes have no affinity for the column surface.

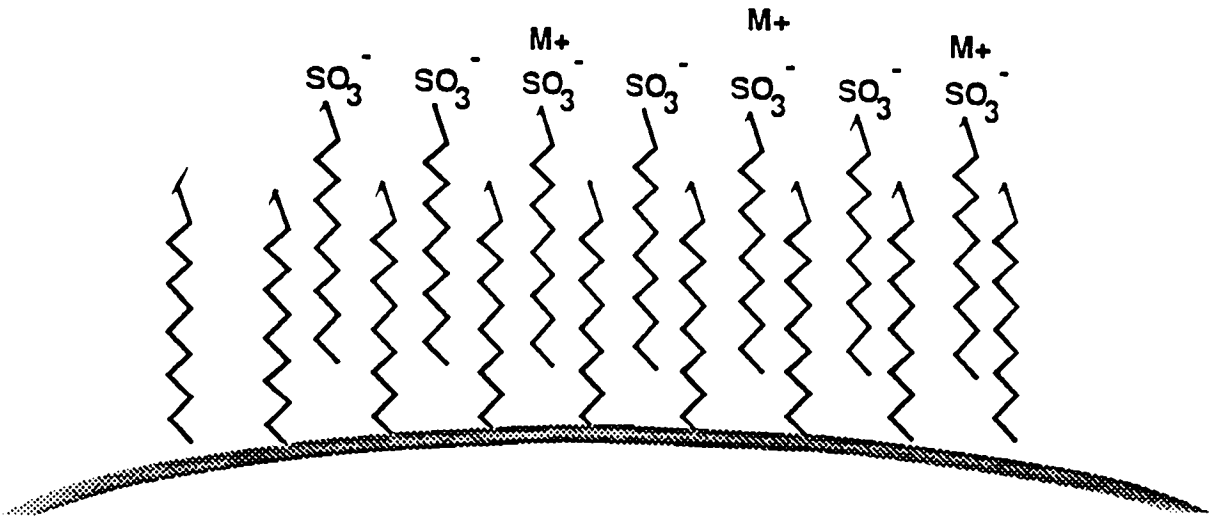
Transition metal selectivity is controled by the following general equilibrium,

$$\text{Org Acid}^- + \text{TM}^+ \rightleftharpoons \text{TM(OrgAcid)}$$

The stronger the complex the earlier the the elution.

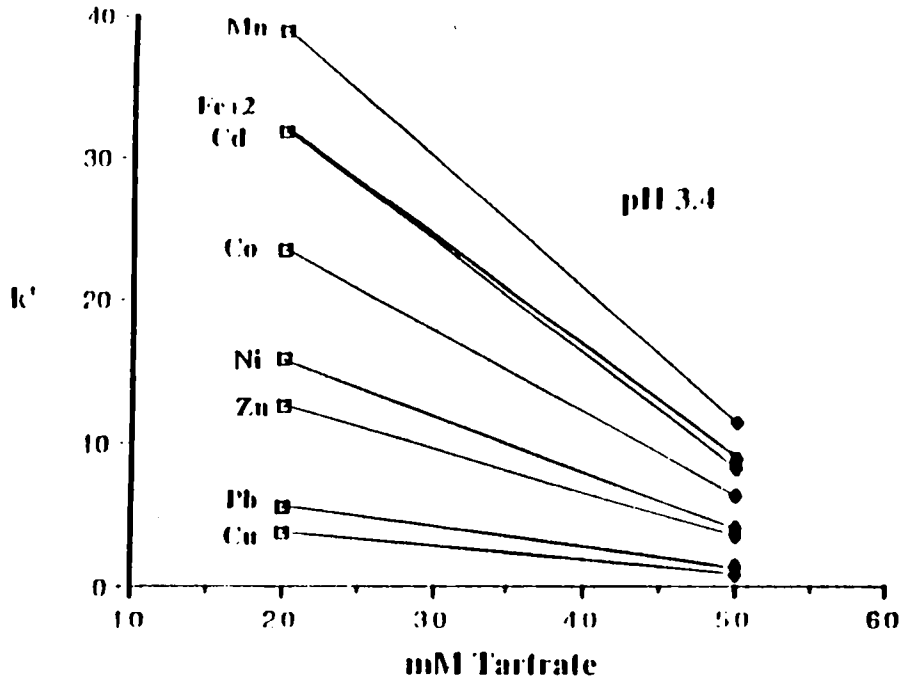
Transition Metal retention is controled by the following
- -Concentration ofOrganic Acid
- -pH of the Eluent
- -Concentration of Organic Solvent

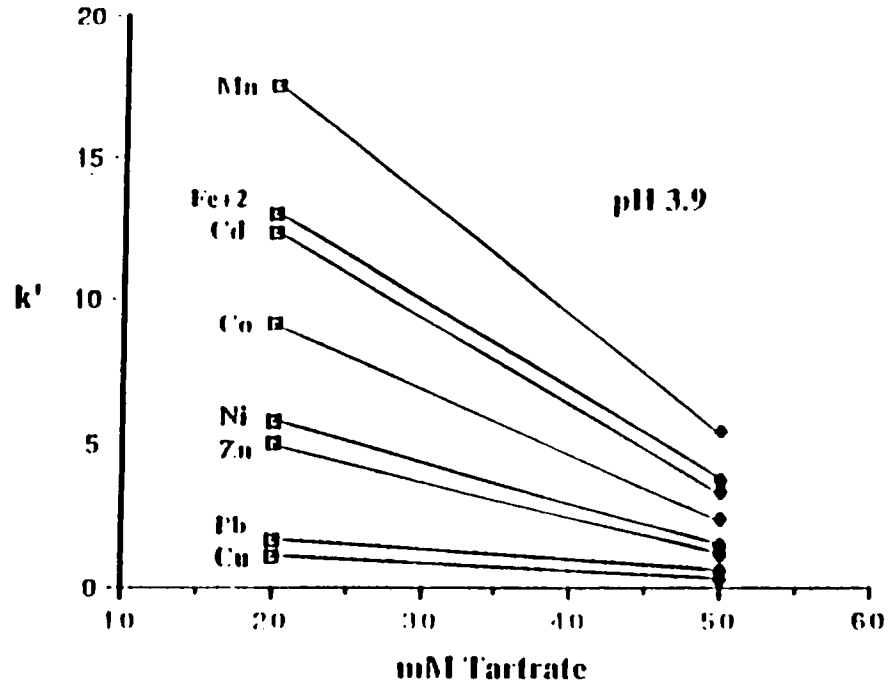




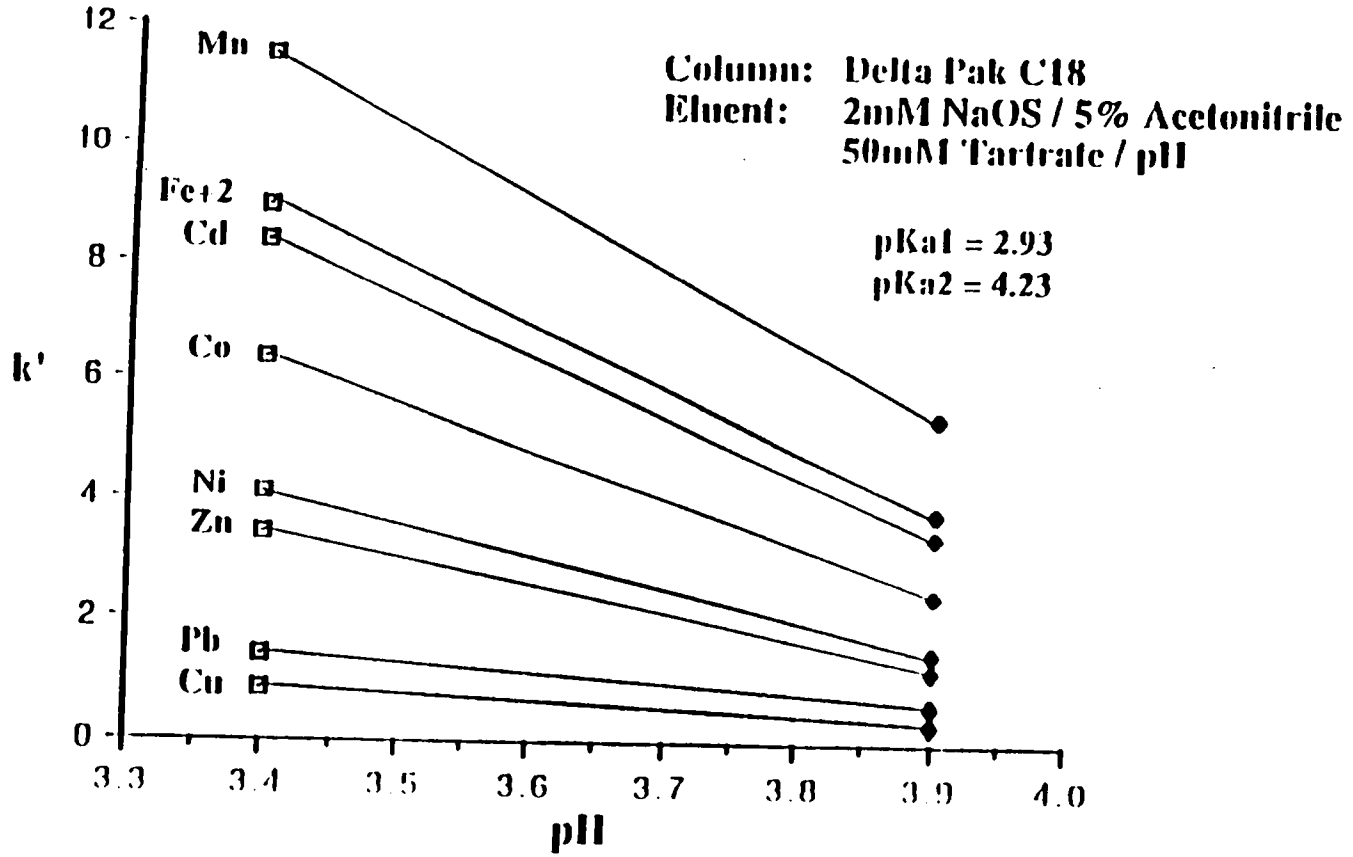
DYNAMIC COATED REVERSE PHASE COLUMNS
TRANSITION METAL ANALYSIS
EFFECT OF ORGANIC ACID CONCENTRATION

Column: Delta Pak C18
Eluent: 2mM NaOS / 5% Acetonitrile
Tartrate / pH 3.4 and 3.9

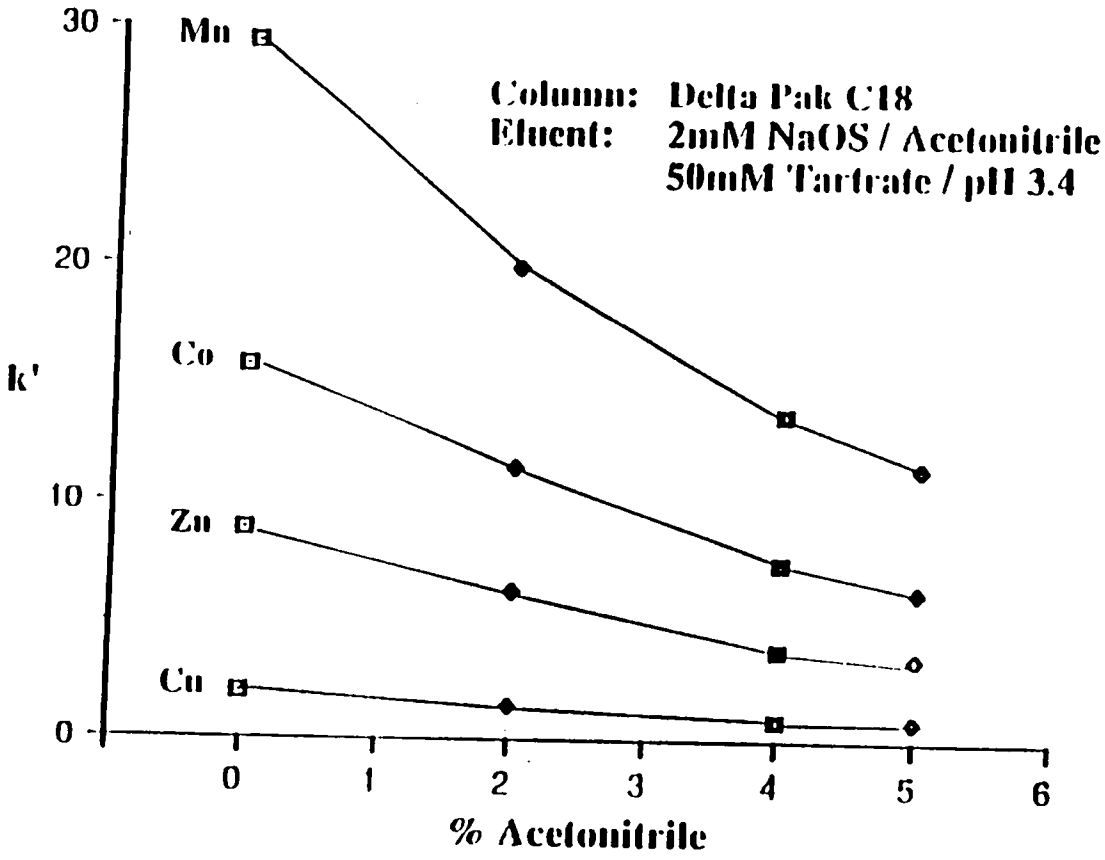




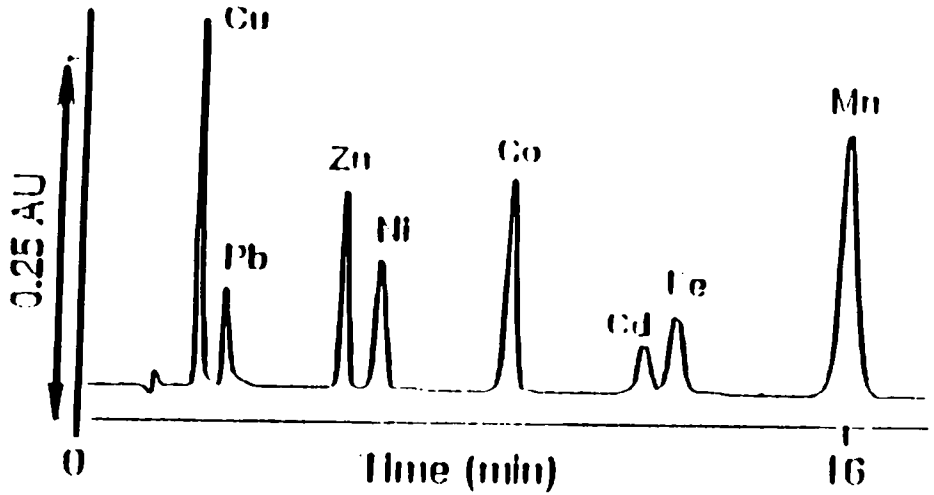
DYNAMIC COATED REVERSE PHASE COLUMNS
TRANSITION METAL ANALYSIS
EFFECT OF pH



DYNAMIC COATED REVERSE PHASE COLUMNS
TRANSITION METAL ANALYSIS
EFFECT OF ACETONITRILE CONCENTRATION



OPTIMIZED
TRANSITION METAL PROFILE
USING DELTA PAK C18



Working Standard

Cu = 0.5 ppm
Pb = 1.0 ppm
Zn = 0.5 ppm
Ni = 1.0 ppm
Co = 0.5 ppm
Cd = 1.0 ppm
Fe = 1.0 ppm
Mn = 1.0 ppm

Method: M-301
Column: Waters Delta Pak™ C18, 100°A
Eluent: 2 mM NaOS , 35 mM Tartaric acid, 5% ACN (pH 3.65)
Detection: UV/Vis at 520 nm, post-column derivatization with PAR

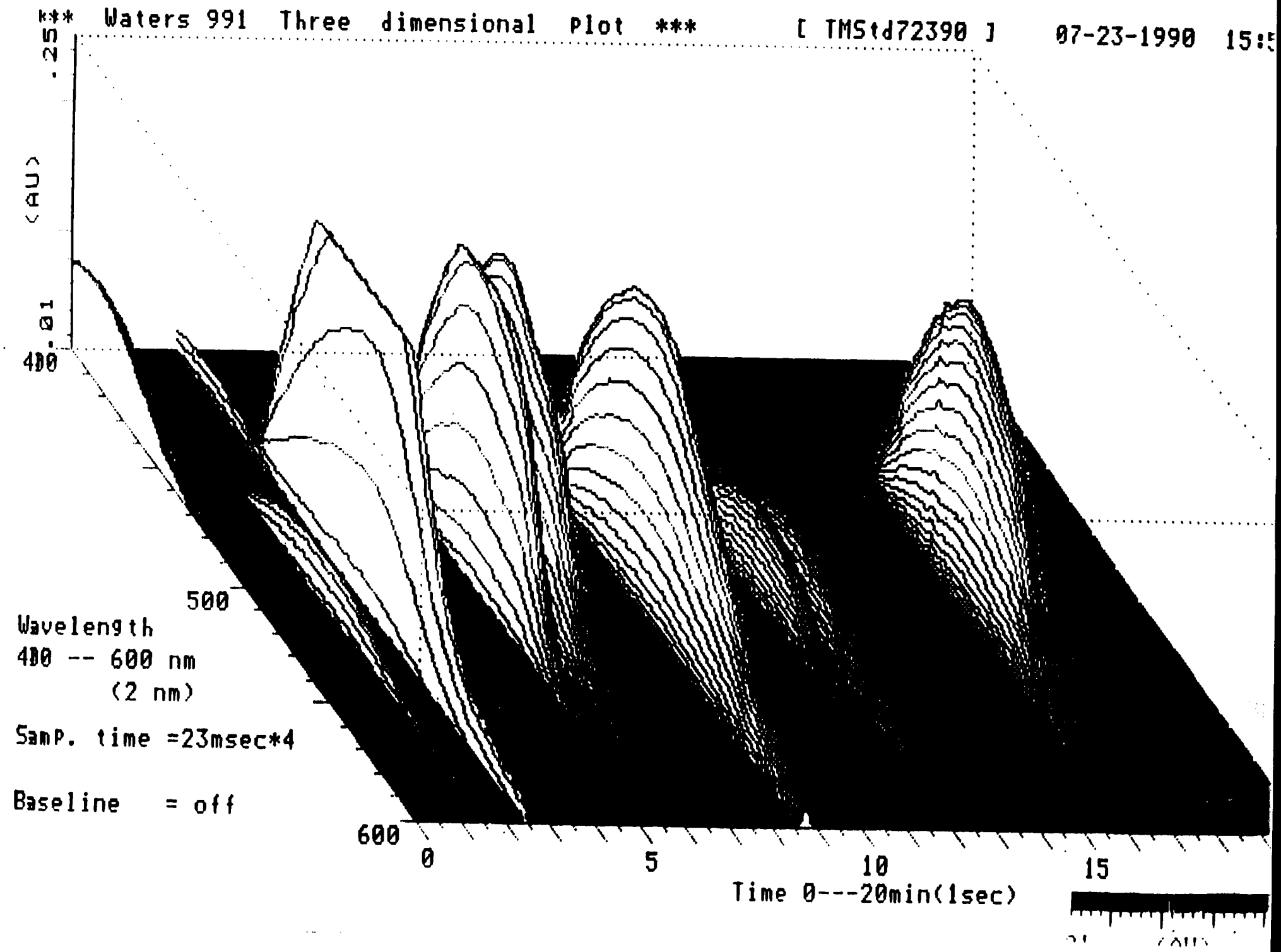
PHOTO DIODE ARRAY ANALYSIS OF PAR-METAL COMPLEX

Shown below is the Waters 991 Photo Diode Array analysis of the transition metal profile using the Delta Pak conditions. Note that PAR-transition metal complex exhibits a broad absorption curve between 490 and 550nm; uncomplexed PAR absorbs in the low 400nm region.

The PAR-transition metal complex is linear between the detection limit and 5ppm; over 5ppm the absorbance exceeds 2AU.

The detection limit using a Waters 490 UV/ Vis detector at 520nm of the highly responsive metals copper, zinc, nickel, cobalt, and manganese is 10ppb with a 100µL injection. The detection limit for iron+3, iron+2, lead, and cadmium is 25ppb.

These detection limits can be improved upon by monitoring each metal at its absorption maxima.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.25
(AU)
.01
400
500
600
Wavelength
400 -- 600 nm
(2 nm)
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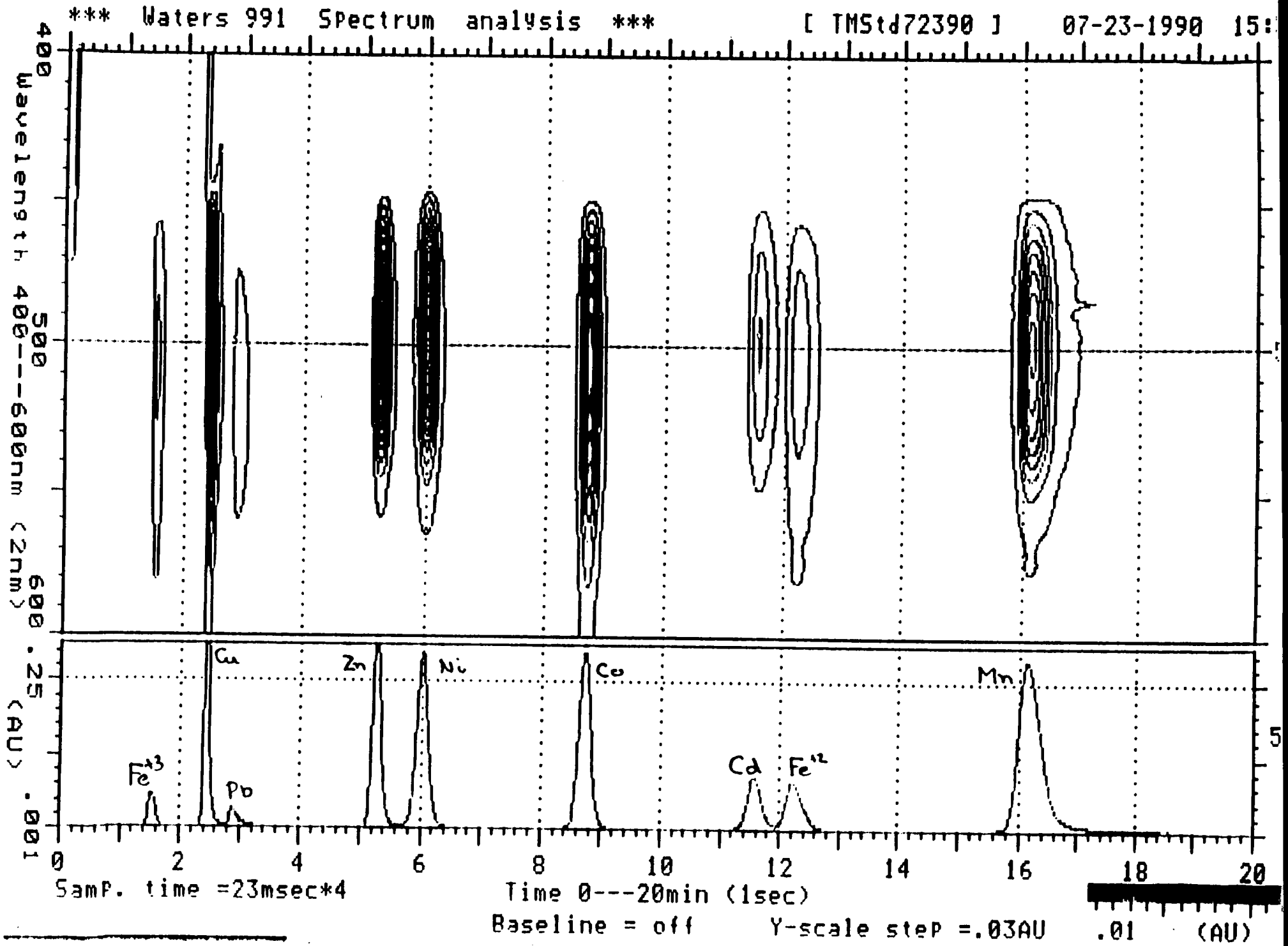
Baseline = off
0
5
10
15
Time 0---20min(1sec)


*** Waters 991 Spectrum analysis ***
[TMStd72390]
07-23-1990 15:
Wavelength 400---600nm (2nm)
400
500
600
.25
(AU)
.001
Fe+3
Pb
Cu
Zn
Ni
Co
Cd
Fe+2
Mn
0
2
4
6
8
10
12
14
16
18
20
Samp. time =23msec*4
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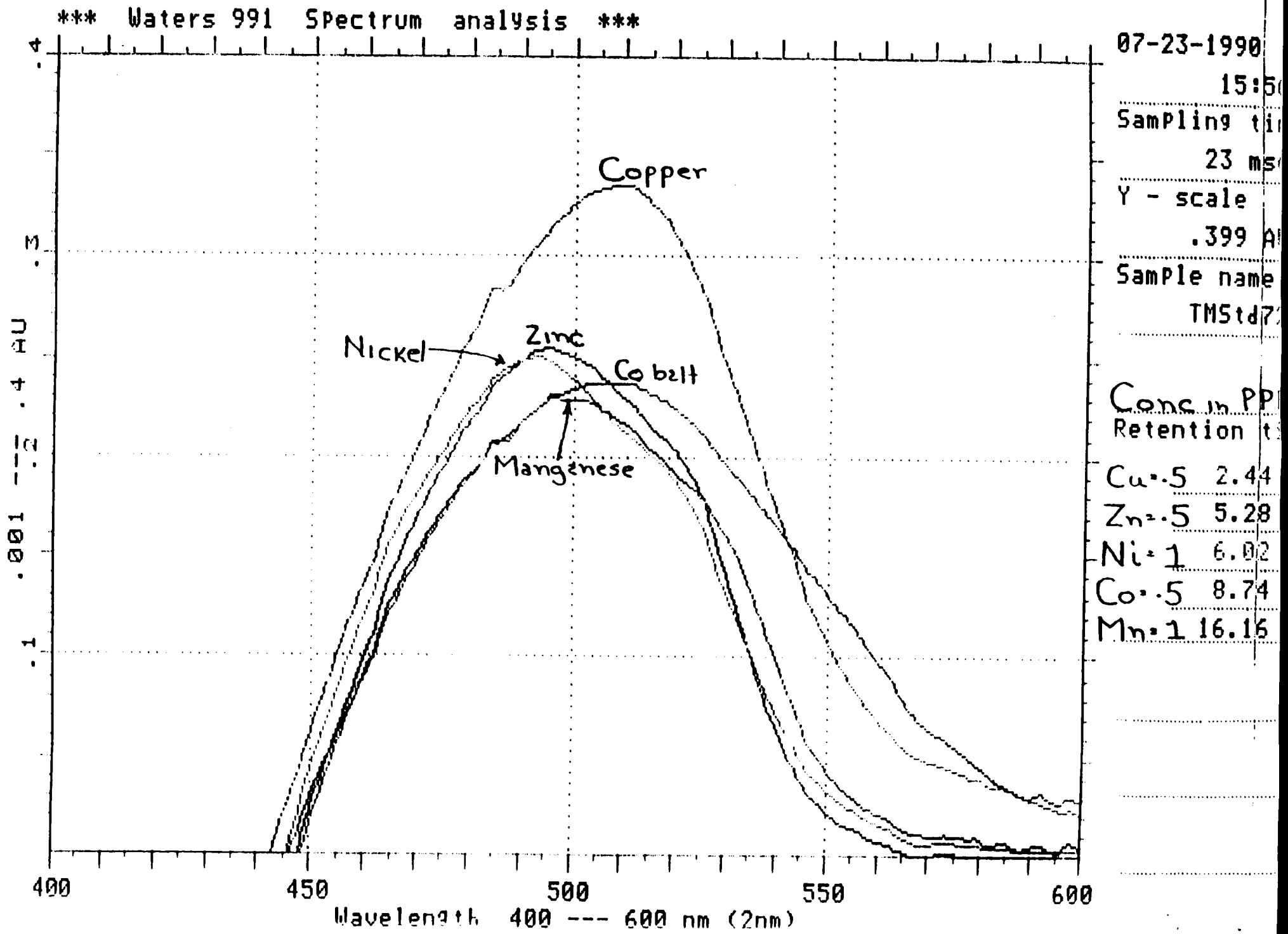
Baseline = off
Y-scale step =.03AU
.01
(AU)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15:5
Sampling ti
23 ms
Y - scale
.399 A
Sample name
TMStd7
Conc in PP
Retention t
Cu=.5 2.44
Zn=.5 5.28
Ni=1 6.02
Co=.5 8.74
Mn=1 16.16
Copper
Nickel
Zinc
Cobalt
Mangenese
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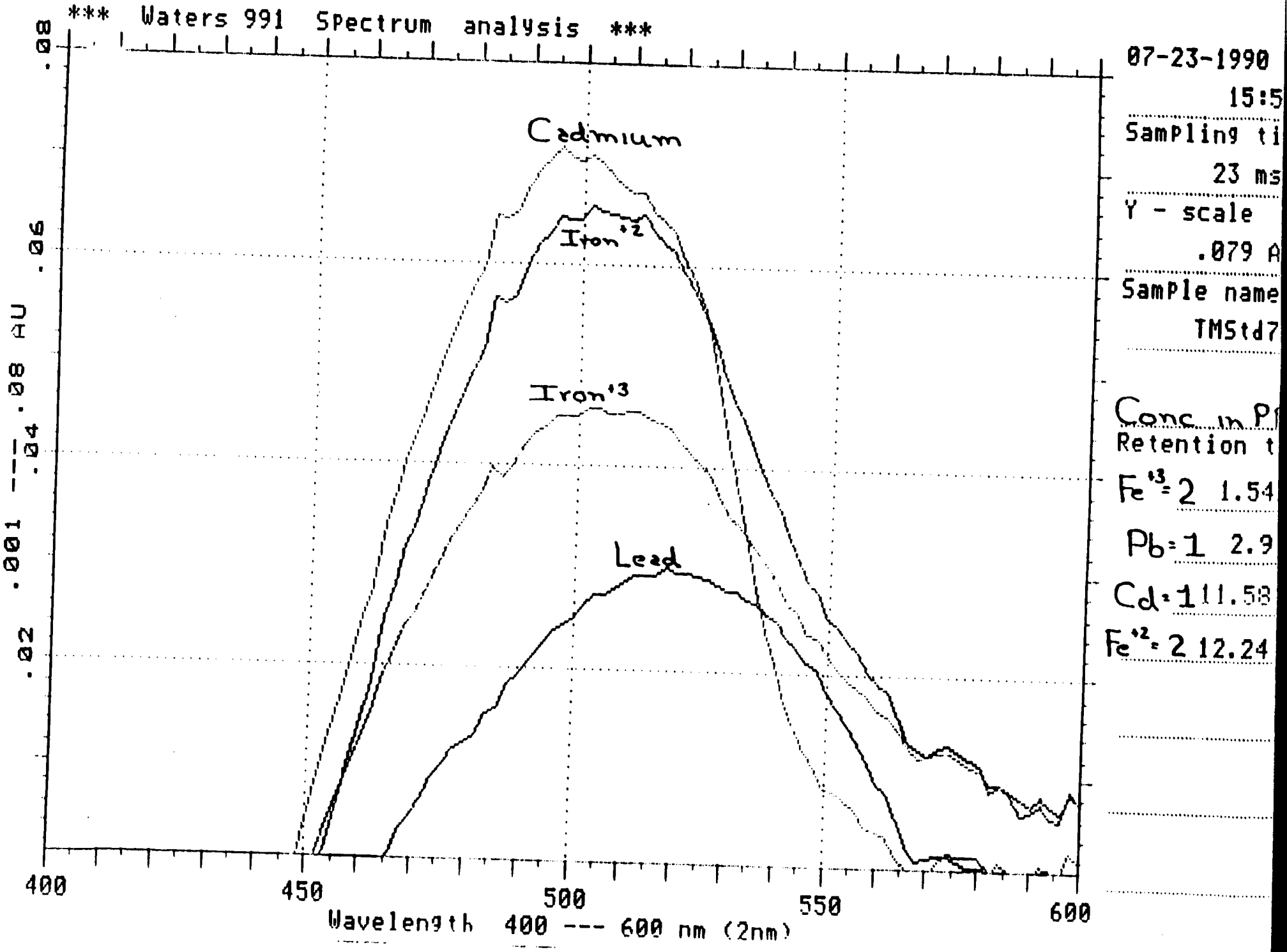
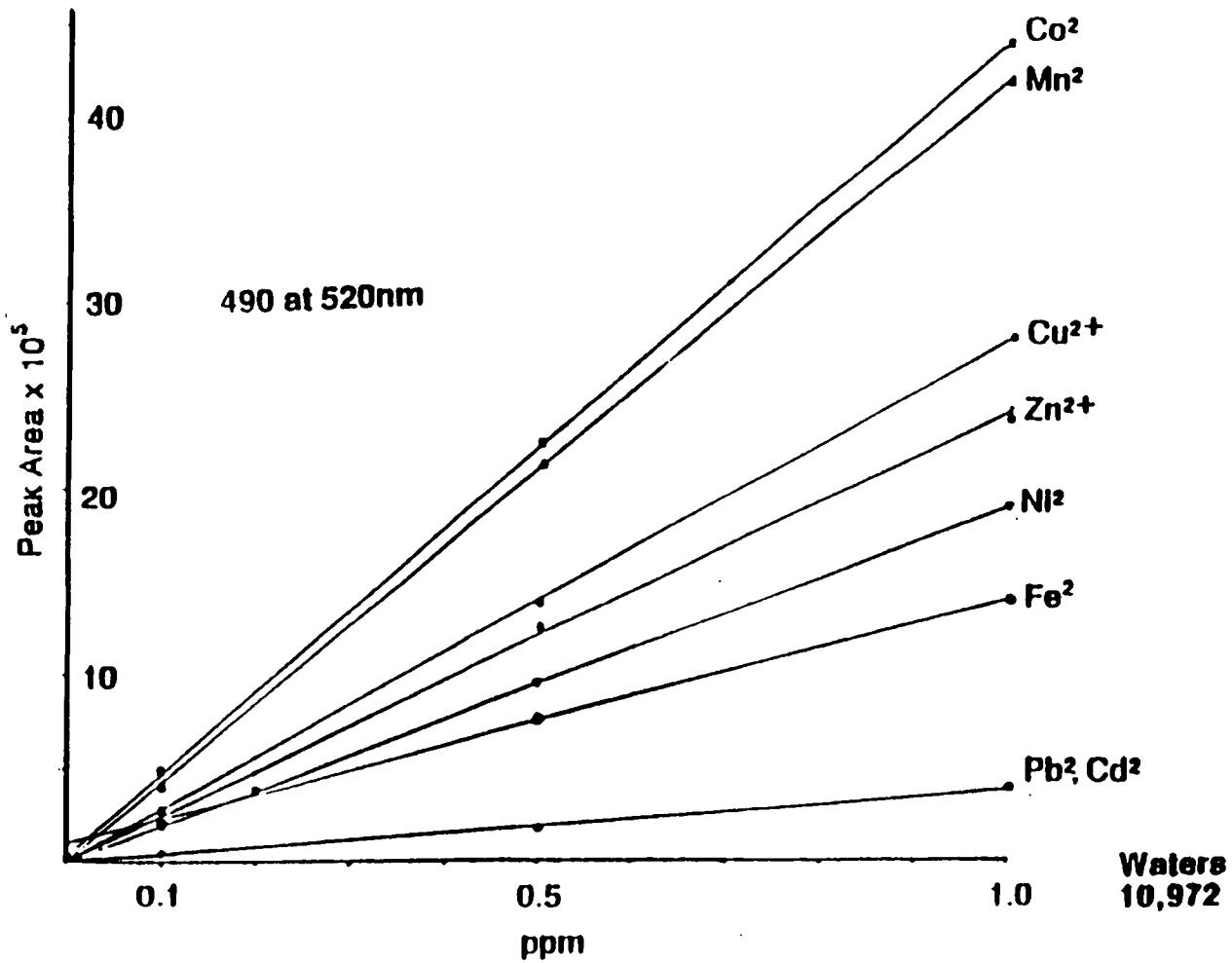
.1
.2
.3
.4
400
450
500
550
600
Wavelength 400 --- 600 nm (2nm)


*** Waters 991 Spectrum analysis ***
07-23-1990
15:5
Sampling ti
23 ms
Y - scale
.079 A
Sample name
TMStd7
Conc in P
Retention t
Fe+3=2 1.54
Pb=1 2.9
Cd=1 11.58
Fe+2=2 12.24
Cadmium
Iron+2
Iron+3
Lead
.08
.06
.04
.02
.001 --- .08 AU
400
450
500
550
600
Wavelength 400 --- 600 nm (2nm)

Transition Metal PAR Response Linearity



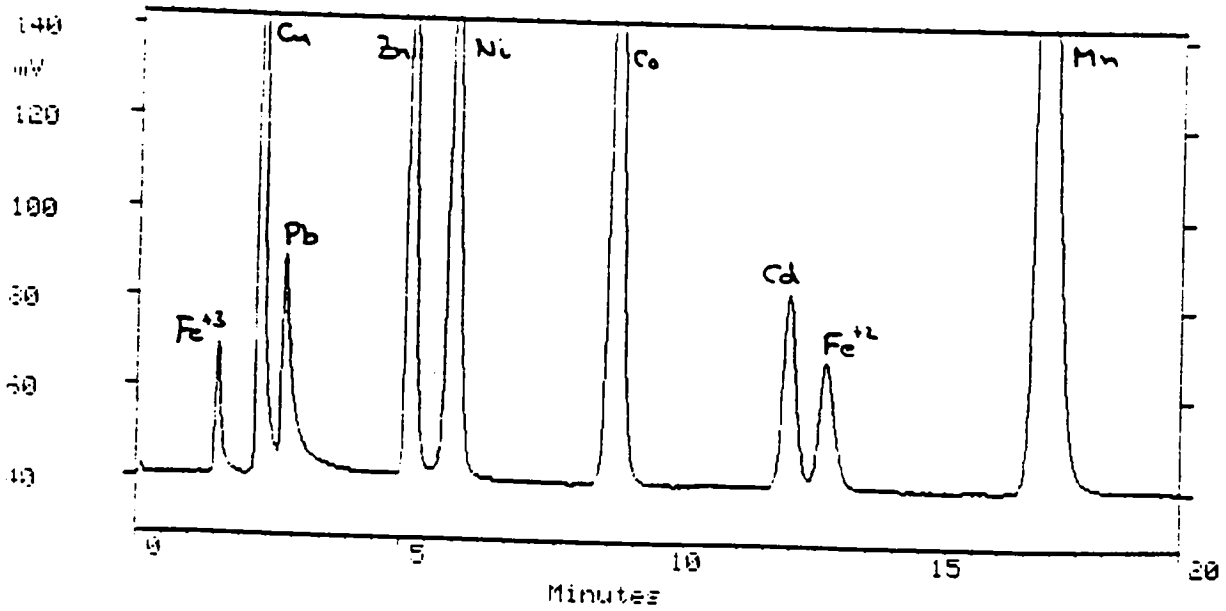
ANALYSIS OF IRON+3

Using this method iron +3 elutes in the void, a region where other strongly chelated metals may elute. Iron+3 can be quantitated as the difference between total iron and iron+2 which is a well retained peak free of interferences.

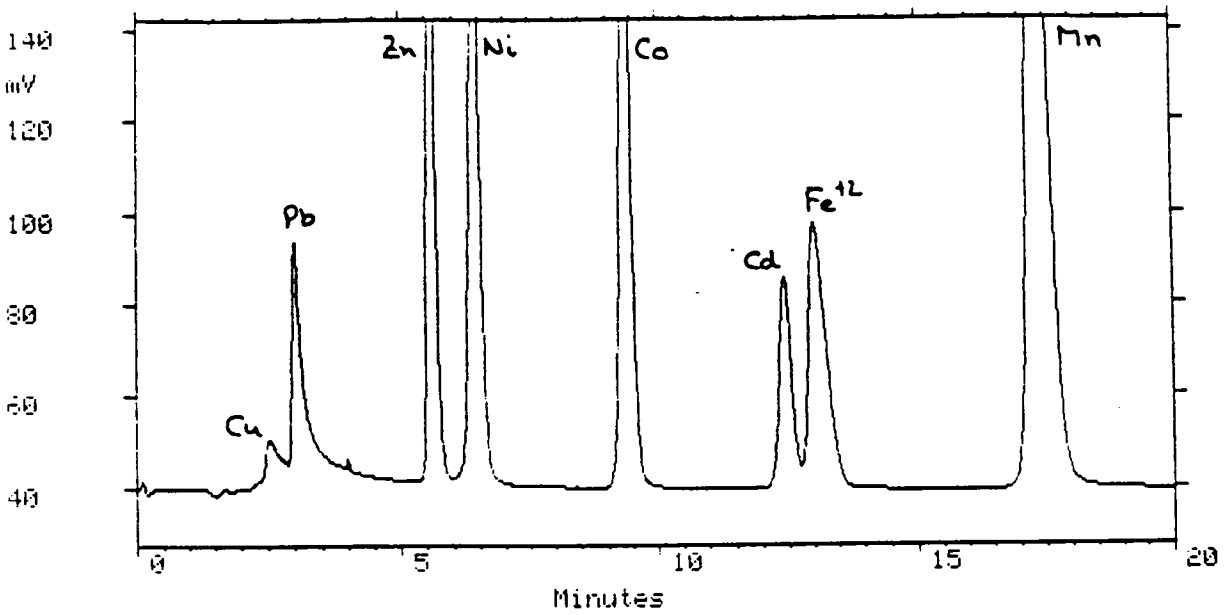
The sample is analyzed for iron+2 concentration. Then 50µL per 10 mls of 5% ascorbic acid is added to the sample; this reduces the iron+3 to iron+2. The sample is injected to determine total iron.

Shown below is the typical transition metal standard before and after addition of ascorbic acid. Note that ascorbic acid does effect the copper peak shape, however, subsequent injections show that copper peak shape returns.

Transition Metal Standard, No Ascorbic Acid

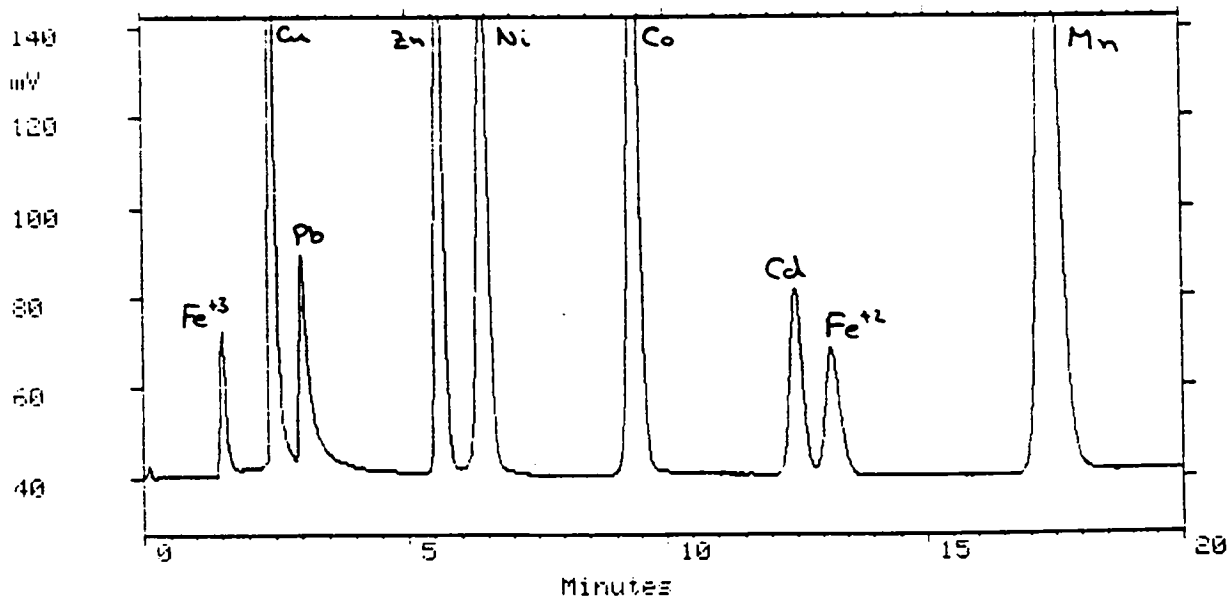


Transition Metal Standard With Ascorbic Acid Addition



Transition Metal Standard ~~Allow~~ NO Ascorbic Acid

NEXT INJECTION



EFFECT OF ORGANICS IN 18 MEGOHM WATER

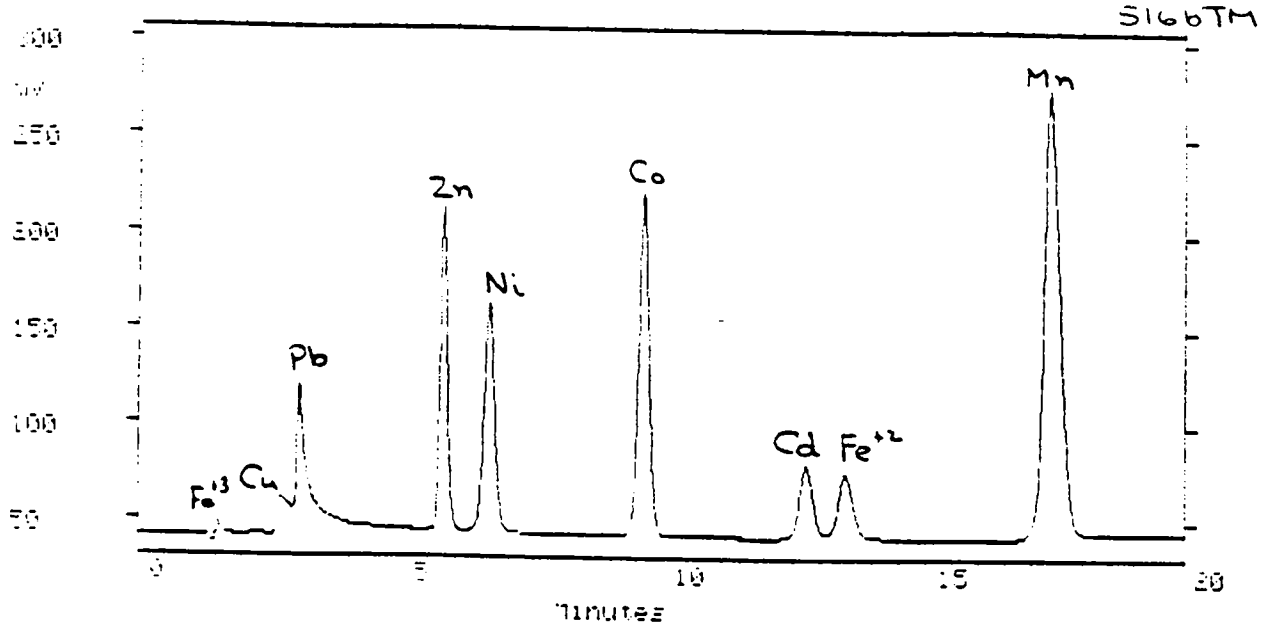
The transition metal method employs the use of C18 reverse phase columns. With the Delta Pak eluent, any neutral organics present in the DI water will tend to concentrate at the head of the column. The organics usually found in 18 megohm water are polar and as such can act as potential chelation sites for the metals. Copper is the only metal that is effected by these organics; copper peak shape will distort and finally disappear as the concentration of the organics on the head of the column increases. This usually occurs within 1 liter of eluent passed through the column.

This problem can be eliminated by washing the column with 50/50 acetonitrile /water for 15 minutes followed by 30 minutes of equilibration with fresh eluent. A means of preventing this problem is to install a C18 scrubber column, a used Delta Pak C18, between the pump and the injector to remove the organics from the eluent prior to injection of transition metals.

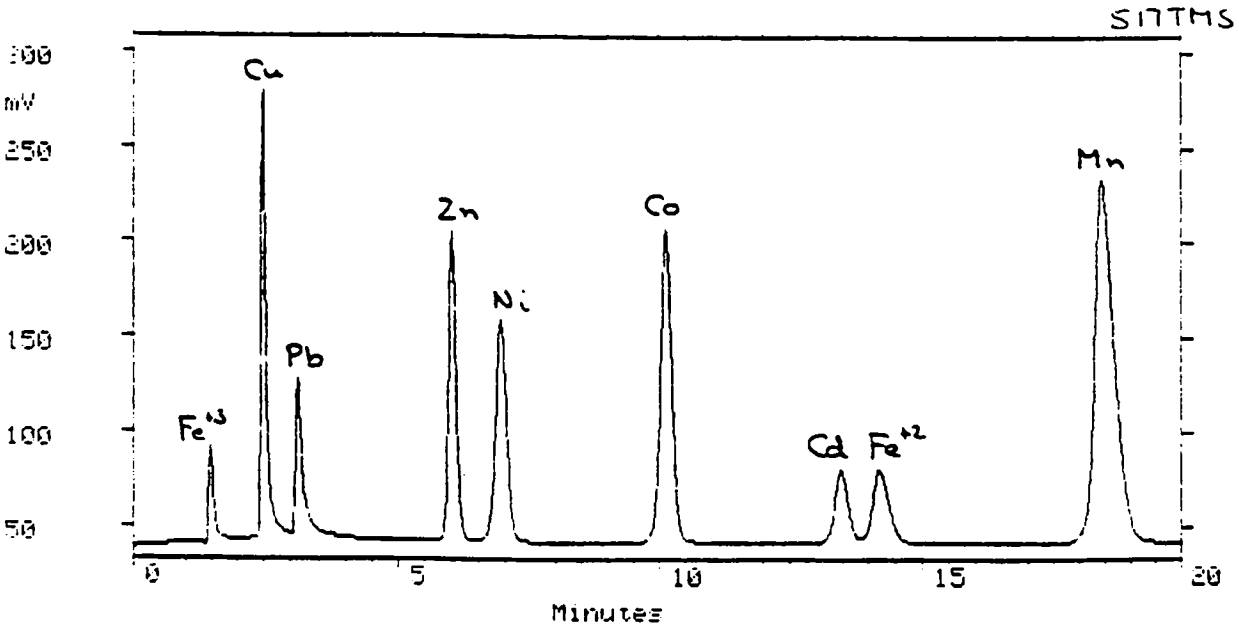
Experience has shown the quality of the 18 megohm water is critical in customer satisfaction. Normally this organics problem is not evident during isocratic reverse phase HPLC or ion chromatography so customer does not believe the problem is with their water. Washing of the column with acetonitrile followed by equilibration with organic free eluent, or by installing the scrubber column, is the best means to demonstrate the problem is with their 18 megohm water.

Shown below are chromatograms showing the effect of organics in the water, and the transition metal profile after acetonitrile wash, and a linear acetonitrile gradient analysis of the contaminated column showing the organics concentrated on the column.

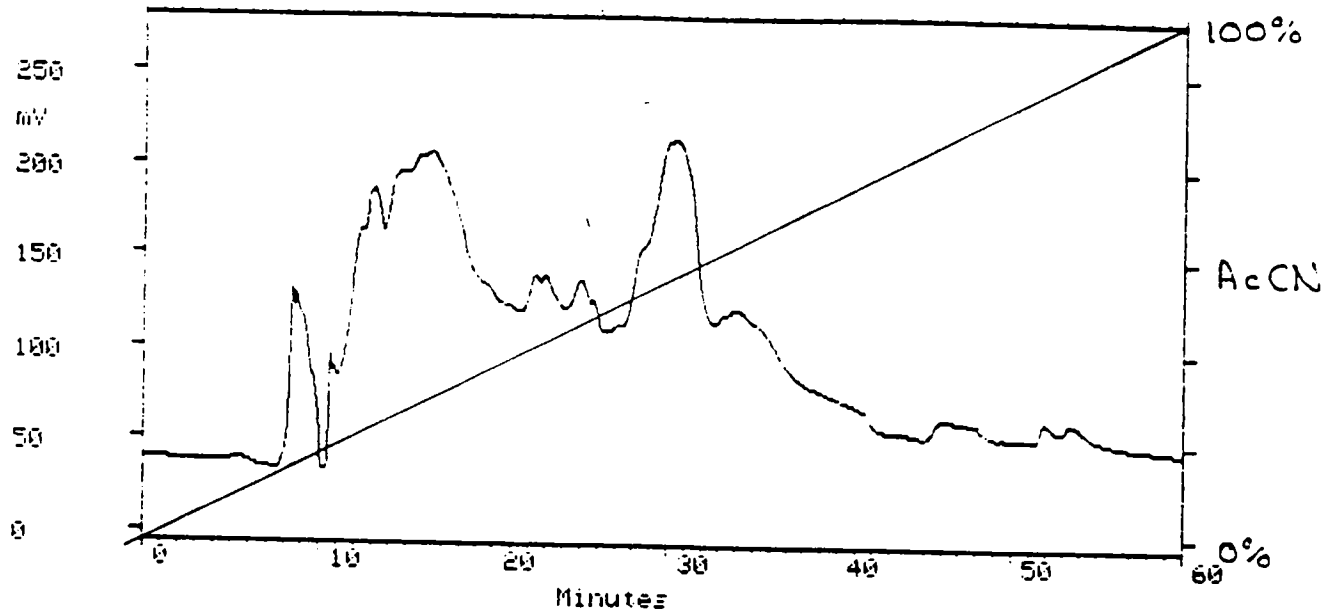
Transition Metal Profile of an Organics Contaminated Column



Transition Metal Profile After Acetonitrile Wash

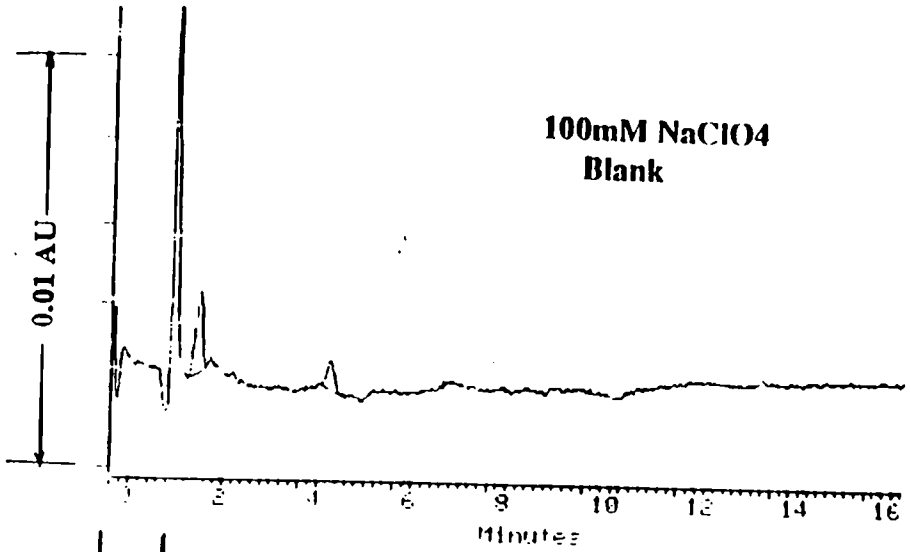


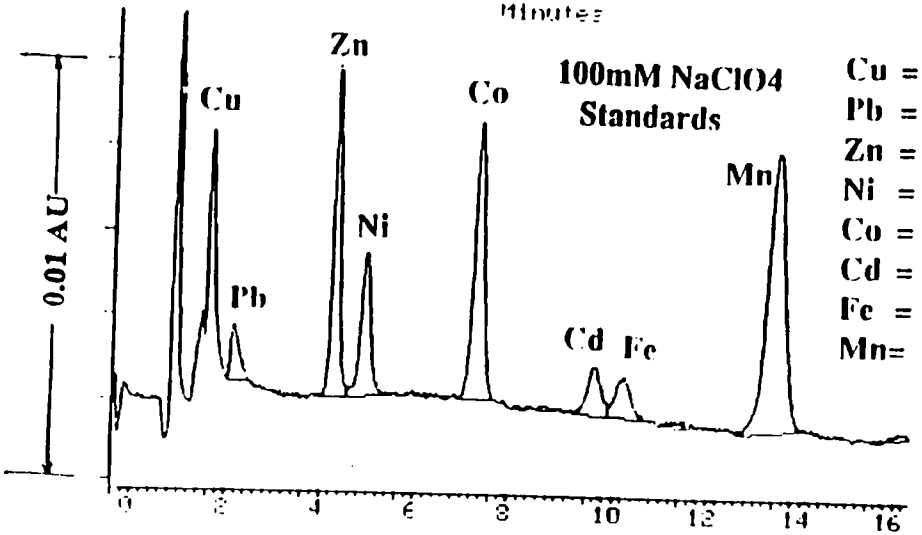
Organics Profile of Contaminated Column Using a Linear Acetonitrile Gradient and Detection at 210nm



HIGH SALT MATRICES

TRANSITION METALS IN NaClO4



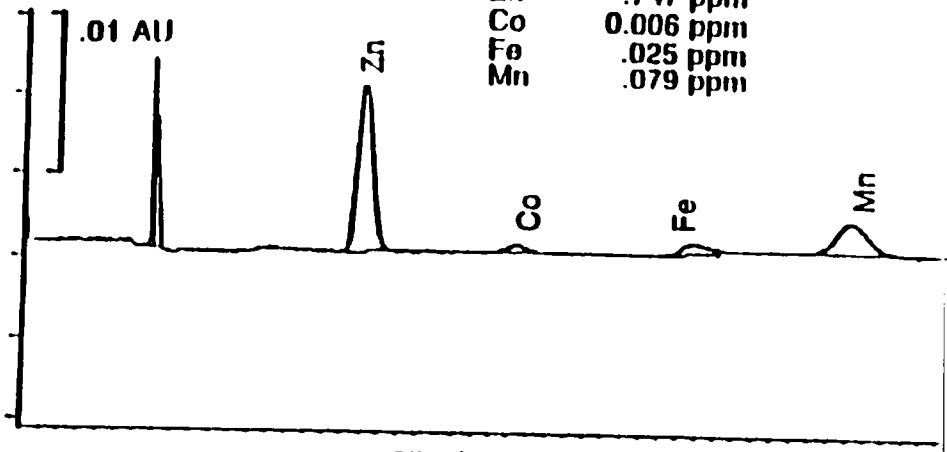


Cu =	25 ppb
Pb =	50 ppb
Zn =	25 ppb
Ni =	50 ppb
Co =	25 ppb
Cd =	50 ppb
Fe =	50 ppb
Mn=	50 ppb

Transition Metal Profile of Crude NaCl Salt

SAMPLE: 1% Crude NcAl in DI Wa
RESULTS:

Zn	.747 ppm
Co	0.006 ppm
Fe	.025 ppm
Mn	.079 ppm



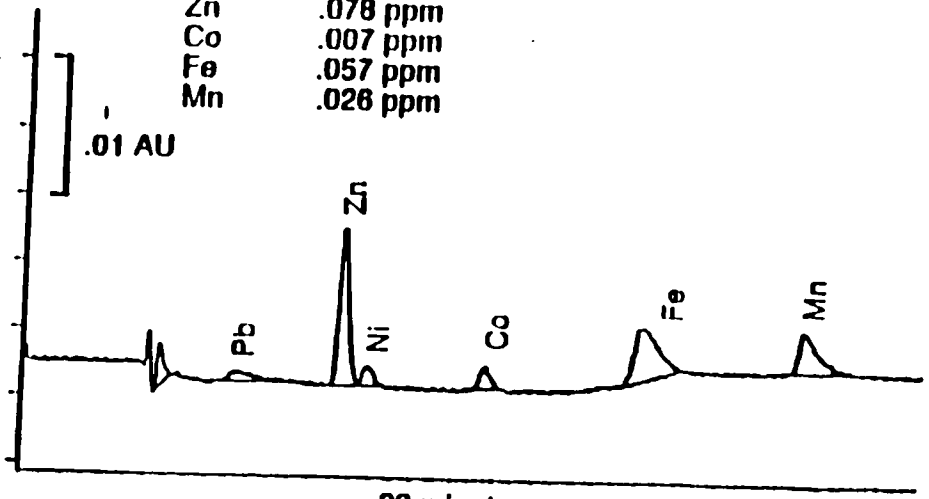
25 minutes

Waters
10,973

Transition Metal Profile of Soil Extract

SAMPLE: 4 grams Soil Suspended in 100 mls 3mM Sr (NO
RESULTS:

Pb	.048 ppm
Zn	.078 ppm
Co	.007 ppm
Fe	.057 ppm
Mn	.026 ppm

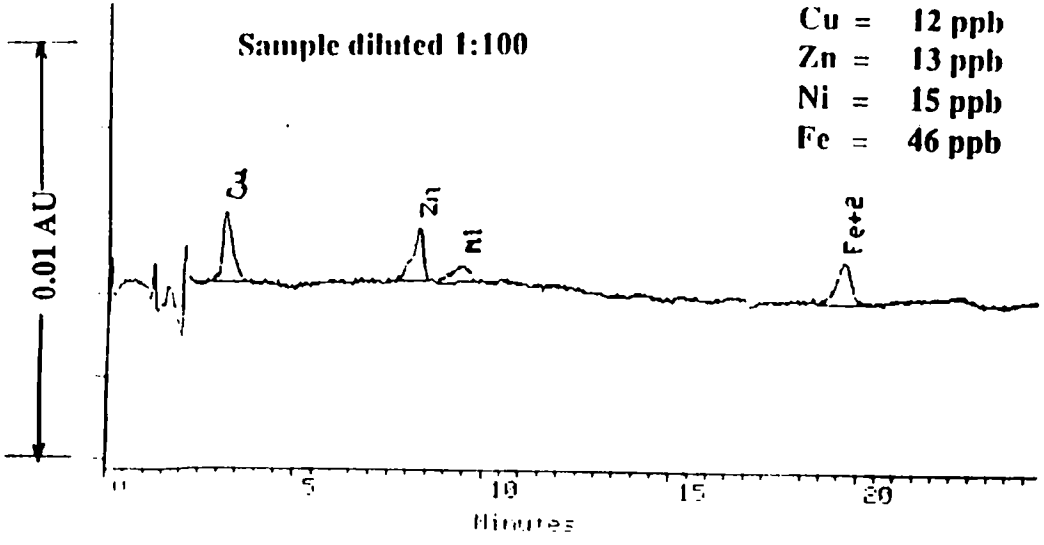


20 minutes

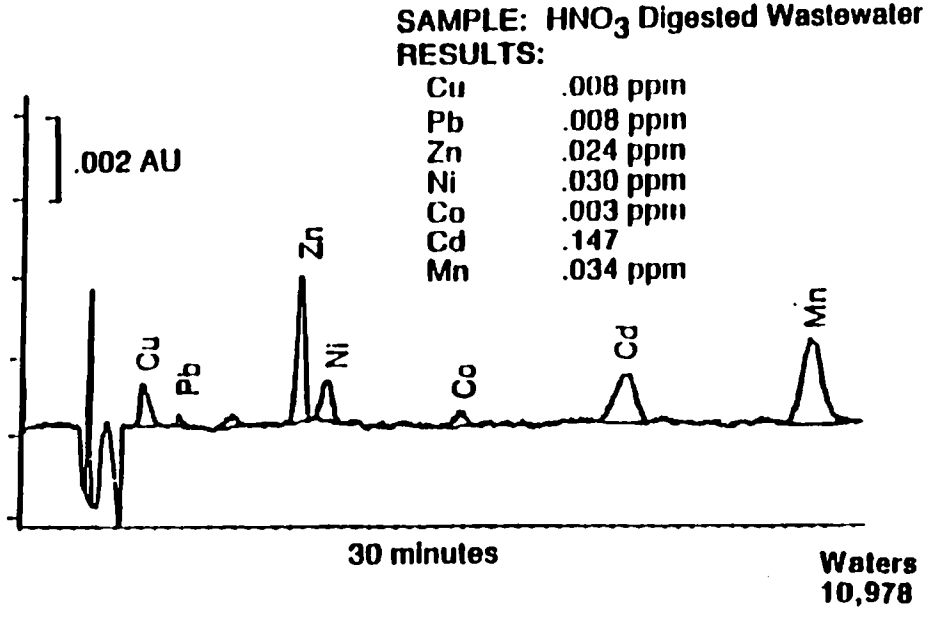
Waters

HIGH ACID MATRICES

TRANSITION METALS IN HBr



Transition Metal Profile HNO_3 Digested Wastewater



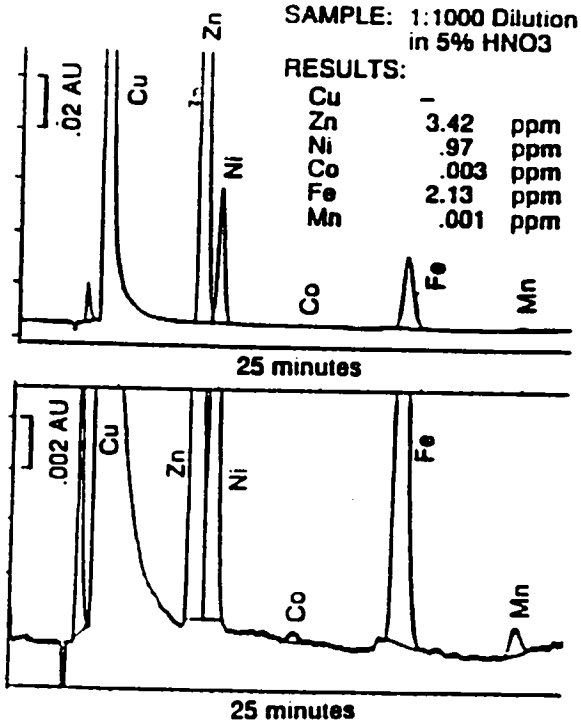
DISPARATE METAL CONCENTRATION

Trace Metals in Acid Copper Plating Bath

SAMPLE: 1:1000 Dilution of Plating Bath in 5% HNO3

RESULTS:

Cu	–	
Zn	3.42	ppm
Ni	.97	ppm
Co	.003	ppm
Fe	2.13	ppm
Mn	.001	ppm

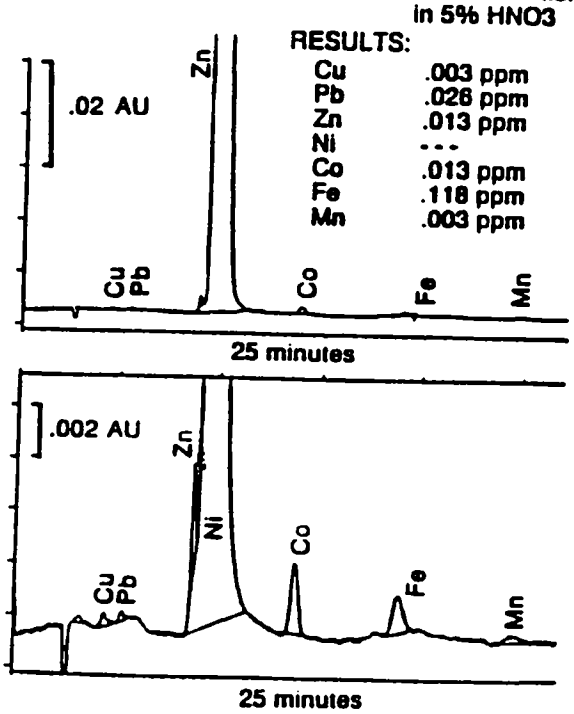


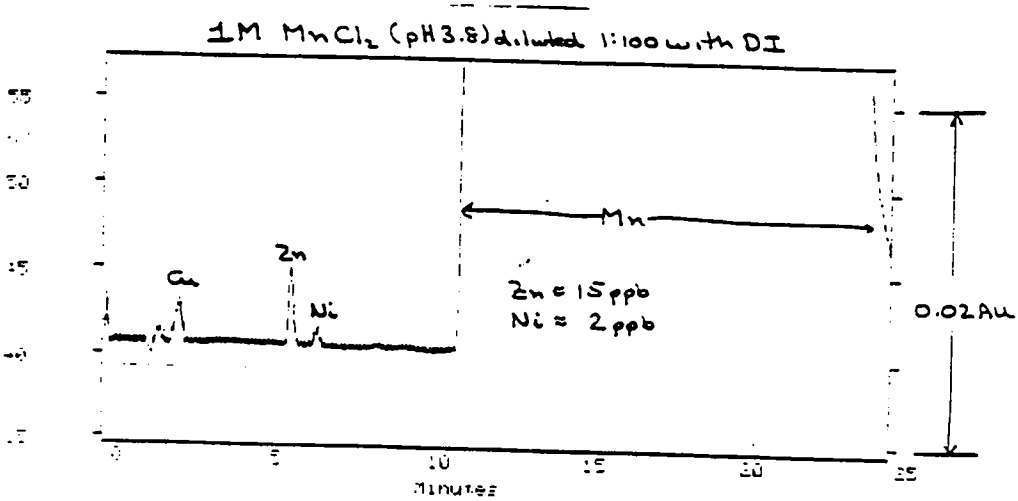
Trace Metals in a Nickel Plating Bath

SAMPLE: 1:1000 Dilution of Plating Bat in 5% HNO3

RESULTS:

Cu	.003	ppm
Pb	.026	ppm
Zn	.013	ppm
Ni	- - -	
Co	.013	ppm
Fe	.118	ppm
Mn	.003	ppm





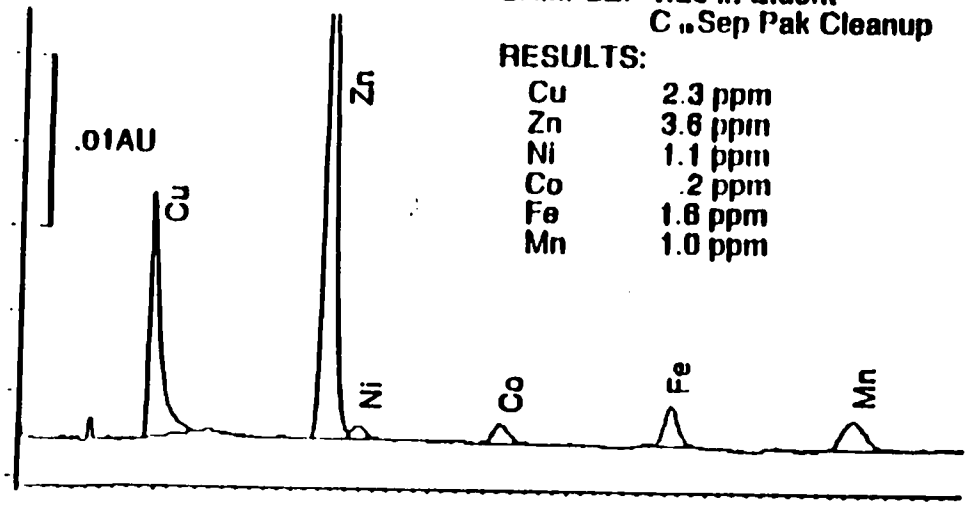
HIGH ORGANIC MATRICES

Transition Metal Profile
Clarified Recombinant DNA
Fermentation Broth

SAMPLE: 1:20 In Eluent
C ₁₈ Sep Pak Cleanup

RESULTS:

Cu	2.3 ppm
Zn	3.6 ppm
Ni	1.1 ppm
Co	.2 ppm
Fe	1.6 ppm
Mn	1.0 ppm

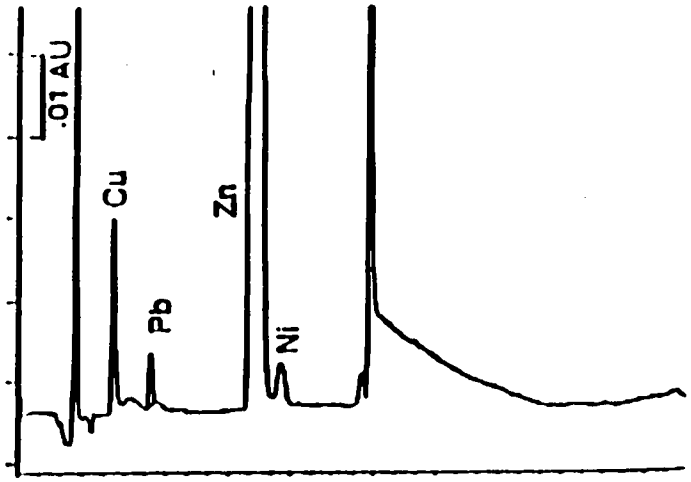


Waters
10,982

Transition Metal Profile
Ashed Milk Resuspended In HNO3

SAMPLE: 100 mls Ashed Milk Resuspended In .5% HNO3
RESULTS:

Pb	.305 ppm



30 minutes

Waters
10,980